IONIC ASPECTS OF THE PHYSIOLOGY AND BIOLOGY OF *MACROBRACHIUM ROSENBERGII* (DE MAN) 1879.

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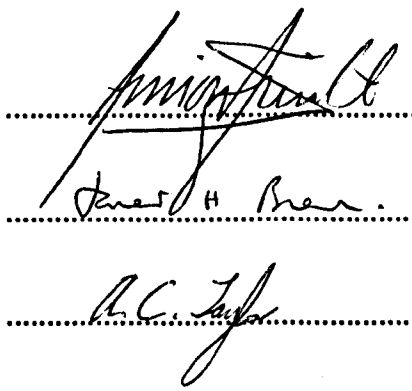
I declare that this thesis has been composed by myself and that it embodies the results of my own research. It has neither been submitted nor accepted for any other degree.



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To Mum, Dad and Zoe

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ABSTRACT

The giant Malaysian freshwater prawn, *Macrobrachium rosenbergii* spends its juvenile and adult life primarily in freshwater. The larval stages of this species require brackishwater for their survival and development. Adult females of *Macrobrachium rosenbergii* migrate into brackishwater to spawn, and some populations of this prawn live entirely in brackishwater. Other *Macrobrachium* species have larval stages that require brackishwater for development. Some *Macrobrachium* species do not require brackishwater for larval survival and have abbreviated larval development.

The ionic requirements for successful larval development and metamorphosis were studied using a formulated artificial seawater. It was found that trace element impurities, either in the salts used in the artificial seawater, or contained within the artemia fed to the larvae, were sufficient to allow normal larval development. The omission of bromide from the artificial seawater was found to cause total mortality to early stage larvae. Further work attempted to discover the minimum threshold concentration of bromide required by the larvae.

The manner by which *Macrobrachium rosenbergii* adults regulate the ionic composition of their haemolymph when exposed to freshwater and brackishwater of varying salinity was investigated. The effect of the moult cycle on divalent cation regulation is studied. It was found that in varying salinity adult *Macrobrachium* showed a strong regulation of its haemolymph osmotic pressure, Na, Cl, Mg, Ca, K and Sr concentrations. The haemolymph Ca and Br concentration increased with increasing salinity, while the haemolymph Cu concentration decreased. The high concentration of bromide and strontium in the haemolymph of prawns held in freshwater was regarded as an indication that they might be essential to the adult prawn. The implication of their roles was in the process of cuticle hardening. A close relationship between haemolymph strontium and magnesium concentrations was revealed, although the significance of this is uncertain.

The effect of the moult cycle on haemolymph divalent cation regulation revealed that Ca and Cu decreased after the ecdysis. Haemolymph magnesium concentrations were elevated during pre- and postmoult. Strontium increased markedly prior to ecdysis, this was further evidence as to a potential role in the calcification

process. The haemolymph bromide concentration was reduced during pre- and postmoult. This was attributed to, either its incorporation into the cuticle, or increased ionic fluxes during the pre- and postmoult period.

The calcification of the larval stages of *Macrobrachium rosenbergii* was investigated. The larvae are calcified in a similar manner to the postlarvae. Concentrations of strontium and bromine in the postlarvae decrease when they are transferred to freshwater. Analysis of larval exuviae revealed high concentrations of bromine (≈1000ppm) confirming its role in the sclerotisation of the larval cuticle. A close relationship was found between the strontium and magnesium concentrations of the larvae and postlarvae held in seawater, confirming this discovery in the adult haemolymph. Such a relationship was not found between these ions and calcium.

Salinity did not affect the function of *Macrobrachium rosenbergii* haemocyanin significantly. This was attributed to the relatively stable internal ionic environment that this prawn is able to maintain over a wide range of salinities. Temperature had a pronounced effect on the haemocyanin oxygen affinity. The oxygen transporting characteristics of *Macrobrachium rosenbergii* haemocyanin were similar to those found for marine and brackishwater crustaceans. The PO_2 of *Macrobrachium rosenbergii* haemolymph was found to be substantially lower than the PO_2's recorded for marine and brackishwater species. This was attributed to a reduced perfusion of the gills by haemolymph. The reason for this was supposed to be a means by which this prawn reduces its permeability, and hence loss of ions, when in freshwater.

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CHAPTER 1 - Background

1.1 Introduction

Freshwater prawns have long been prized as a delicacy in South-East Asia where there are important fisheries for a variety of species (Rabanal & Soesanto 1985). Fisheries for the various *Macrobrachium* species exist in tropical and sub-tropical countries all over the world. In the late 1950's, due to an increasing demand for freshwater prawns and declining wild catches, attention was turned to the possibility of culturing these species particularly the Giant Malaysian freshwater prawn, *Macrobrachium rosenbergii.* The problem that was encountered was that whilst the adults grew well in freshwater, the larval stages died soon after hatching. This inability to close the culture cycle limited the aquaculture of this species to the ongrowing of wild caught postlarvae. The discovery that the larval stages required saline water for successful development was made by S. W. Ling, and he subsequently detailed the methods for the production of postlarvae (Ling 1969a,b). It is unfortunate that Ling has received all the credit for this discovery, since previous authors had hinted at the necessity of saline water in the life-cycle of this prawn due to the absence of larvae in freshwater trawls, but their presence in brackishwater. John (1957) is quite emphatic about the requirement for saline water and mentions that successful moulting of the stage I to stage II larvae occurred in saline water.

The landmark papers of Ling (1969a,b) on the biology and culture of *Macrobrachium rosenbergii* were the first steps towards the freshwater prawn industry of today (Sandifer & Smith 1985; Rabanal & Soesanto 1985; New 1988; Hsieh *et al.* 1989; New 1990). Once established, the growth of freshwater prawn farming was extremely rapid, particularly in Thailand and Taiwan. In 1976 production of *Macrobrachium rosenbergii* in Thailand was only 3 metric tonnes per year (t/yr), by 1979 this had grown to 80 t/yr, and in 1987 production was estimated at 15,000 t/yr (New 1988). Taiwanese production of *Macrobrachium rosenbergii* in 1982 was 250 t/yr out of 250 ha of ponds. This increased to 4,500 t/yr in 1988 with an estimated 3,500 ha of ponds in production (Hsieh *et al.* 1989). Global production of freshwater prawns is estimated at 27,000 t/yr, of which Thailand, Vietnam and Taiwan produce

44%, 32% and 17% respectively. The development of the culture industry for the various *Penaeus spp.* has been even more spectacular, and this has tended to overshadow the culture of freshwater prawns. In 1989 production of *Penaeus monodon* was estimated at 80,000 t in Thailand alone. The advantages of *Penaeus monodon* culture are its faster growth rate and the ability to rear this species at high density. This has led to environmental problems in recent years, but production still remains very much greater than that of freshwater prawns, which account for only 5% of global shrimp and prawn production (New 1990).

The various *Macrobrachium* species do have certain advantages in other respects. The lower stocking density that has to be employed with these species results in a slower degradation of the pond environment, and allows the use of feeds with lower protein contents. This is thought to be due to supplemental feeding by the prawns on benthic fauna. A second advantage is that *Macrobrachium spp.* can be reared inland in freshwater. Thus, if a tropical or sub-tropical country has large freshwater resources, but little access to the sea, prawn culture may still be feasible. However, there is still an important constraint; the larval requirement for saline water. If commercially viable postlarval prawn production can be achieved in these areas, then there is considerable potential for the production of this high value species.

1.2 Biology

The genus *Macrobrachium,* a member of the family Palaemonidae, comprises approximately 125 species, which are found in tropical and sub-tropical environments worldwide (Holthuis 1949, 1950a, 1950b, 1980). Species within this genus are found in freshwater, brackish and occasionally marine habitats. Although Hedgpeth (1949) states that there are no marine species, Holthuis (1980) lists one species (*Macrobrachium intermedium*) that has its main habitat in the sea.

Ortmann (1902) suggested that the Palaemonidae are a relatively recent family currently in the process of invading freshwater. His evidence for this is that representatives are widely found in the marine environment, but that there are also species that have brackish or freshwater habitats. Some palaemonid species may have

become secondarily marine. Panikkar (1941) suggests that species such as *Palaemon serratus* and *Palaemon elegans* may have returned to a marine habitat having successfully invaded fresh or brackishwater. The evidence for this is the strong osmoregulatory ability of these species. Panikkar argues that their ancestors successfully penetrated freshwater by developing the necessary powers of osmoregulation and reducing their permeability, and then subsequently reinvaded brackish and marine habitats. His second point concerns the low isosmotic point of these species. If they had never left the marine environment the isosmotic point could be expected to be higher. However, the euryhalinity of the palaemonid prawns may simply be a feature of this family that has allowed them to invade brackish and fresh waters.

This recent invasion of freshwater from the sea is also demonstrated by the requirement for saline water for larval development in most of the freshwater Palaemonidae (e.g. *Macrobrachium rosenbergii,* Ling & Merican 1961; Johnson 1967; Ling 1969a,b) Only a few of the 125 known species of *Macrobrachium* have fully broken this link with the sea and are able to complete their entire life cycle in freshwater (e.g. *M. australiense, M. potiuna, M. ihrengi, M. borelli, M. shokitai*). Those that live in freshwater but require brackishwater for larval development must make a migration downstream towards the sea to spawn, and thus ensure that their larvae develop in brackishwater.

The larvae of all palaemonid prawns hatch as a zoeal stage. However, the duration of larval development and number of stages varies. There are three basic types of larval development in the *Palaemonidae* (Sollaud 1923). Firstly, those marine and brackishwater species which require brackishwater or seawater for larval development. Such species are characterised by producing numerous small eggs and by having an extended larval life involving numerous stages. Secondly, those species which inhabit freshwater and whose larvae can develop in freshwater. These species produce a small number of large eggs and have a shortened larval development. Thirdly, there are a few freshwater species in which larval development proceeds entirely within the egg and the animal hatches at the post-larval stage. *Macrobrachium rosenbergii* is an exception to this scheme, as the adults inhabit freshwater, yet produce numerous small larvae that require brackishwater for development.

The variation in number of larval stages and development times is

exemplified by the following freshwater *Macrobrachium* species:

1) *Macrobrachium borelli* - The larvae hatch directly into postlarvae (Boschi 1961).

2) *Macrobrachium hendersonayanum* - has one larval stage before metamorphosis to postlarva (Jalihal & Sankolli 1975).

3) *Macrobrachium shokitai* - has two larval stages, but takes only 30 hours to metamorphose to postlarva (Shokita 1973b).

4) *Macrobrachium australiense* - has three larval stages (Lee & Fielder 1984).

5) *Macrobrachium rosenbergii* - has 11 (Uno & Soo 1969) or 18 (Gomez-Diaz & Kasahara 1987) larval stages, and takes anywhere between 18 and 50+ days to reach postlarva. The larvae require brackishwater for development.

Early studies showed that larval decapod crustaceans were principally planktotrophic and carnivorous (McConaugha 1985). Decapod larvae usually pass through a number of larval stages prior to metamorphosis into a postlarval stage. The duration of this larval period varies greatly between species, as does the number of larval stages. There is a trend in some estuarine and especially in freshwater decapods to increase the extent of embryonic development and thereby reduce the number of free living larval stages needed to reach the postlarval form. There is also a move towards lecithotrophy, thereby reducing the larval requirement for planktotrophy. The advantages of this are clear especially with respect to the wholly freshwater species. The freshwater habitat is particularly unsuitable for planktotrophic larvae, this is principally due to the much lower concentration of plankton in this environment (Magalhaes & Walker 1988). This in turn is due to the general difference in the reproductive strategies of marine and freshwater species, and to the effect of downstream displacement in a riverine environment. Thus if the larva of a species were to hatch in a plankton poor environment it would have to survive on a yolk reserve until such time that it was able to feed. A larval decapod that hatched in a morphologically advanced condition might be able to survive on a yolk reserve until it metamorphosed into the postlarval form (e.g. *Macrobrachium shokitai* Shokita 1973b, *Macrobrachium asperulum* Shokita 1977). Any food encountered could be consumed but the absolute requirement for food could be offset by a larger yolk reserve. In order that a larva can be endowed with this extra reserve of yolk, to allow greater embryonic development and lecithotrophic larval stages, the egg must be larger.

The increased investment of energy and essential dietary components in these larger eggs inevitably means that the fecundity of the animal is reduced. This is seen in many species that have extended embryonic development. The reduction in fecundity and increase in egg size seems to be a feature of those *Macrobrachium* species which have penetrated furthest into freshwater. Indeed there is some evidence that intraspecific differences can arise between populations in the same river system. Mashiko (1990) gives evidence of differences in egg size and number between estuarine, riverine and landlocked populations of *Macrobrachium nipponense.* He does not suggest that there are differing numbers of larval stages, but it is possible that the freshwater populations have a greater investment in the larval yolk reserve. This could be to allow the larvae to survive longer as they are washed downstream to brackishwater where they spend their larval phase. This difference in yolk investment is significant as this species has planktotrophic larvae that live in brackishwater. This suggests that the capacity to increase the yolk content exists within a species should its habitat make this desirable, alternatively, this feature is selected for in the up-river populations. Mashiko (1984) also reported that two populations of *Macrobrachium nipponense* living in the same river system, one up-river, the other estuarine, did not interbreed. He concluded that this was principally due to geographical isolation. It might be that the sheer distance of migration for the up river population is such that the larvae are released by the female before brackishwater is reached. This would mean that the larvae have to exist for a period of time in freshwater, living off the yolk reserve, before entering the estuarine environment where they could commence feeding.

Macrobrachium rosenbergii is a species whose larvae hatch as non-feeding zoea. This stage I larva survives on its yolk reserve until it moults into the stage II larva and starts to feed on plankton. If the stage I larva hatches into freshwater it will not moult to the stage II and so it does not feed, but continues to use its yolk reserve. The reason why the stage I larva does not moult in freshwater will be considered later. The stage I larva can survive for five days in freshwater (Ling 1969). Thus it would seem that this species is adapted for the possibility of hatching in freshwater and being subsequently carried into the estuarine environment.

There is a problem apart from nutrition which confronts the larvae of freshwater decapod crustaceans that hatch in freshwater, and this is the maintenance of

body fluid composition and concentration. It may well be that this is the principal reason why so many species have larvae that are confined to brackishwater. Adult crustaceans have well developed powers of osmotic and ionic regulation and specialised organs to perform these functions (e.g. the gills and antennal glands). The larvae of marine decapod crustaceans have functional antennal glands but lack gills (Foskett 1971). This is not the case in later stage larvae of *Macrobrachium rosenbergii*, which have gills clearly visible beneath the cephalothoracic carapace.

The larval stages of *Macrobrachium petersii* (Read 1984) osmoregulate over quite a wide range of salinities, and the larvae of *Macrobrachium rosenbergii* also have a wide salinity tolerance, 5-20 °/oo (George 1969). Thus the estuarine environment is suitable for both food and as a maintenance medium. The optimal rearing salinities for the commercial species of *Macrobrachium* are all between 10-15 °/oo. It would seem therefore that larval osmoregulation presents a considerable barrier to any palaemonid species that is trying to break its link with the sea. The stage I larvae of *Macrobrachium rosenbergii* (Ling 1969), *Macrobrachium faustinium* (Hunte 1980), *Macrobrachium carcinus* (Choudhury 1971) and *Macrobrachium petersi* (Read 1984) can all tolerate freshwater on hatching, but none will moult to the second stage. This lack of moulting appears to be the key to the ability of stage I larvae to survive in freshwater. Once the stage I larvae moult to stage II they are no longer able to tolerate freshwater and die within 30 minutes if exposed to it. Subsequent larval stages are also unable to tolerate freshwater. This ability is only regained when the larvae metamorphose to postlarvae.

One of the behavioural features of *Macrobrachium spp.* postlarvae is that they are benthic and positively rheotactic. This means that once they have metamorphosed they will always move upstream. This is in contrast to the larval stages which tend to be planktonic, although they hide amongst vegetation and floating material such as twigs and leaves (Wehrtman & Dittel 1990). Thus, planktonic larvae will tend to be displaced downstream. There is, therefore, another positive advantage in having a short larval life for those species which live high up in a river system and which do not require brackishwater for larval development since the young of that species are less likely to be displaced from the adult habitat. Thus it would seem that the complete adaptation to freshwater by some *Macrobrachium* species involves a diminution of the larval phase. This is enabled by the supply of a large yolk reserve, and greater development within the egg.

Behavioural mechanisms exist in adult *Macrobrachium* to ensure that their larvae hatch into the correct environment. Female *Macrobrachium australiense* become positively rheotactic when spawning. This causes them to maintain their position in the river or to move upstream. Thus when the larvae hatch they are carried by the water flow into the habitat that the female left. The short larval life does not allow much time for the larvae to be carried downstream. As soon as they metamorphose into postlarvae they begin to move upstream again. This species has an entirely freshwater life cycle.

Those species which still have brackishwater larval development have a completely different rheotactic response on spawning. The females of *Macrobrachium rosenbergii* and *Macrobrachium acanthurus* (John 1957; George 1969; Hughes & Richard 1973) migrate downstream towards the sea. It does not matter if they do not reach the sea since their larvae can tolerate freshwater for a number of days as they are carried downstream. Once they have released their larvae, the females become positively rheotactic again and begin to migrate back up-river.

The key to the tolerance of freshwater by the stage I larvae of these species that have brackishwater development is suggested to be in the permeability of the larvae. Read (1984) argues that if the ionic permeability of the stage I larvae was less than the successive stages, then all it has to regulate is the osmotic influx of water when in fresh or low salinity water. When transferred from seawater to freshwater the stage II larvae of *Macrobrachium rosenbergii* rapidly turn white and die (Sandifer & Smith 1975, pers. obs.). This is without having moulted and thus it would seem that the cuticle is allowing the larvae to gain or lose some vital factor(s) when placed in freshwater. The cuticle of the stage I larva is formed within the egg where it is protected from the rigours of the freshwater environment, and it is possible that some factor is provided by the female that is required for its formation. This would be available to the stage I larvae but may not be readily available to successive stages of larvae, or be energetically difficult to concentrate from freshwater. However, this factor may be readily available in the marine environment. Panikkar (1941) suggests that those species that have a reduced larval life, or that have complete larval development within the egg, must invest all the nutritional and physiological elements the developing larvae will require. He concludes by stating that this is a principal requirement for the successful invasion of freshwater by egg bearing species. Those species which have brackishwater larval development, but a

freshwater adult habitat are considered to lack the necessary powers of osmoregulation in the larval phase. The ability to osmoregulate is thought by Panikkar to develop at metamorphosis.

The transition between the brackish and freshwater environments exposes *Macrobrachium rosenbergii* to widely differing physical and chemical environmental conditions. Whilst the adults and stage I larvae can tolerate freshwater, the other larval stages cannot. The intention of this thesis is to try and elucidate the extent to which *Macrobrachium rosenbergii* has adapted to life in freshwater, and what physiological changes occur in its transitions between the freshwater and brackishwater environments. To this end it was proposed to study aspects of the adult physiology in fresh and saline water. The larval requirement for seawater for successful development prevents this species from completing its lifecycle in freshwater. This is of considerable importance in the culture of this species, since it limits the culture of postlarvae to areas that have reasonable access to the sea. It was thus proposed that the possible culture of larvae in artificial seawater would be attempted, with the hope of elucidating what physical or chemical aspects of seawater were necessary for correct larval development.

CHAPTER 2 - Larviculture of *Macrobrachium rosenbergii.*

2.1 History and commercial methods

The aquaculture of freshwater prawns has only developed to its current scale by having an abundant supply of postlarvae for the stocking of ponds. Seed production can often be a serious limiting factor in aquaculture operations either because of inadequate numbers or prohibitive cost. Whilst the major expenditure in a growout operation is feed cost, the seed cost can be a substantial percentage of that production cost. Thus there is a need for a cheap and simple method for the production of postlarvae. Fujimura & Okamoto (1970) developed the first mass culturing method of *Macrobrachium rosenbergii* postlarvae, and the technique they described has remained largely unchanged to the present day in many hatcheries.

The method of Fujimura & Okamoto is now known as 'greenwater culture', so called because of the use of phytoplankton to improve the water quality and nutrition of the larvae. Gravid females at approximately the same stage of egg development are placed in large concrete or fibreglass tanks containing brackish water (salinity 6-12 ppt). The water in the tanks contains phytoplankton which can be derived from three sources:

1) It can be cultured as single species in bottles and small tanks, which are then used to 'bloom' phytoplankton in the concrete culture tanks. This is generally the more difficult method of 'greenwater culture' because the blooming of unialgal cultures involves a degree of hygiene, the use of algal culture facilities and the maintenance of stock cultures.

2) Tilapia, or any euryhaline fish, can be maintained in a tank containing brackish water. The introduction, and feeding of the fish will bring in sufficient algal cells to allow the water to bloom. The feeding of the fish, and their excretory products, provides the nitrogenous compounds required by the phytoplankton to bloom. Once the tank has developed a rich phytoplankton bloom, the water can be removed to the larval culture tanks. Filtered brackishwater replaces that removed and the dilution effect prevents the bloom from becoming too dense. If this were to happen there is the possibility of a collapse

('crash') in the phytoplankton bloom.

3) Nitrogen and phosphate containing fertilizers can be added to outdoor tanks containing brackish water. The phytoplankton present will boom and the water can then be removed to the culture tanks. Maintenance of the algal bloom can be quite difficult, therefore several tanks need to be maintained.

The phytoplankton in the larval culture tank are thought to remove nitrogenous metabolites produced by the larvae and their decaying food. Water changes are minimal in the greenwater system, the tanks being gradually filled as the larvae grow. This has the effect of reducing their stocking density as they become larger and more cannibalistic. By keeping the volume of water low in the early stages of culture the amount of feed given can be minimised. The water is generally changed only if the bloom becomes too dense, or the nitrite and ammonia levels become too high. The degree of water quality monitoring varies between hatcheries; often there is none. The greenwater system works most effectively under low stocking densities (10-20 postlarvae/litre), and generally gives unpredictable survival.

The main drawback of the greenwater system is the threat of a phytoplankton crash. If the phytoplankton bloom is very dense its oxygen demand can be very high at night and this can stress the larvae if there is inadequate aeration. In addition, high phytoplankton productivity during daylight hours can give pH values as high as 9.9-10.5. Mass larval mortalities can occur if the pH exceeds 9.5 (New 1990). If the concentrations of nitrogenous metabolites are high this will also stress the larvae. These stressors can then predispose the larvae to disease. If a bloom crash occurs, there is a sudden rise in biochemical oxygen demand (BOD), and the concentrations of ammonia and nitrite. These can kill the larvae in their own right, and any survivors are weakened and susceptible to disease (New & Singholka 1985). There is also the problem of overfeeding; if too much food is placed in a tank the natural decomposition processes that are normally sufficient to maintain water quality are overtaxed. This leads to a water quality problem and can result in disease.

The environmental stresses encountered by the larvae reared using the greenwater culture system mean that they are not as susceptible to these stresses when they are stocked into the growout ponds. Ponds often have problems with low aeration and

poor water quality. If the larvae are not acclimated to these conditions before introduction to the ponds, survival can be poor.

Due to the inherent variability of the greenwater method, efforts were made to establish a method by which *Macrobrachium rosenbergii* postlarvae could be produced without the use of phytoplankton, and at substantially higher larval stocking densities. That is, it was considered desirable to intensify the method of production. As a result of research at the *Centre Oceanologique du Pacifique* (AQUACOP), the clearwater method of larval rearing was established. Early attempts resulted in high mortality rates due to poor water quality. This was caused by the build up of nitrogenous compounds and the high organic loading presented by the feed. The early method was only successful with the repeated application of antibiotics (AQUACOP 1977). This prevented the development of bacteria which would have exploited the organic environment and subsequently infected or fouled the larvae. A problem can arise from the use of prophylactic antibiotics instead of ensuring good water quality, and that is the dosage required to prevent bacterial build-up in the system gradually increases. The use of antibiotics and sometimes sulpha drugs is commonplace in larval rearing, although not widely publicised (New 1990). The problem of the deterioration of water quality in static tanks under high stocking density cannot be resolved unless sufficient water is replaced in the tanks on a daily basis. This is because the high feed concentration and number of larvae causes fouling of the water within 24 hours, thus once-daily changes are inadequate. Even if substantial water changes are given, these cause wide fluctuations in water quality.

An advantage of the clearwater system is that it is a culture system that operates at only one trophic level, that of larvae feeding on their prey. This causes a theoretical simplification of the process as it removes the need for the phytoplankton. However, this system is more labour intensive due to the need for cleanliness and the careful control of feeding. The lack of phytoplankton in this system could affect the nutrition of the larvae. This is because artemia that are unfed utilize their yolk reserves (Szyper 1989). It is possible that the algae could concentrate essential trace elements, or provide fatty acids or amino acids not present in inert larval feeds or starved artemia.

Clearwater systems have been devised utilising recirculation systems, thereby reducing the problem of nitrite and ammonia build-up between daily water changes. However, due to their high running cost they are not widely used , although some

French owned/designed hatcheries utilise coral biofilters at 5% of the larval rearing volume (New 1990). This system involves containing the filter material in mesh bags. At regular intervals half of the bags would be removed for cleaning and be replaced with preactivated bags. In this way the filter never becomes too heavily loaded with organic material, and yet does not suffer from the loss of denitrification caused by the cleaning of the filter (P.Y. Kersuzan pers. comm. 1990). The use of biofilters is rarely viable in commercial culture due to the cost of construction and the constant monitoring they require. Possible exceptions to this are situations where the conservation of heat, or water, changes the economic balance.

If water is a limiting resource then it might be suggested that an aquacultural operation should not be attempted ! However, in the culture of freshwater prawns this is a common constraint. If there is no access to seawater, then no matter how much freshwater is available, the production of prawns is impossible. This is one of the problems that can prevent the establishment of freshwater prawn farms in inland areas with abundant freshwater resources. The requirement of *Macrobrachium rosenbergii* larvae for brackishwater confines their production to the coastal regions of many countries. In Thailand, hypersaline seawater, obtained from coastal salt farms, is carried by tanker to the hatcheries. These are sited by the farms in inland areas (Tunsutapanich 1981). The distance that it is feasible, or economically viable, to carry hypersaline water will vary between locations. The distance the hypersaline water is transported in Thailand is no more than about 100 miles. The rearing technique used by these hatcheries can be either greenwater or clearwater, and requires the use of very large volumes of seawater. Thus, even though the price of the hypersaline water is not high, transportation is, and hence the cost of the seawater increases.

If an area lacks salt farms, or is too far inland to transport water then alternatives to natural seawater must be considered. These alternatives are either defined aquarium salts, reconstituted whole seasalt, or homemade artificial seawaters. All of these have been tried with varying success (Tunsutapanich 1981; Smith *et al.* 1982; Tansakul 1983; Yambot & Vera Cruz 1986). Thus there is a problem with the economically viable mass culture of postlarvae in inland areas. Recirculation systems can reduce the volume of water required, and are able to maintain satisfactory water quality at stocking densities in excess of those possible using the greenwater system. However, they are more expensive and can be difficult to use, but may still be feasible if there is sufficient demand

for postlarvae.

2.2 Larval nutrition

In section 1.2 it was proposed that one of the advantages of having an extended larval life in brackishwater is that it allows the hatching of a relatively underdeveloped larva. This then enables a greater number of larvae to be produced since fewer resources are required to produce them. This section considers how these small larvae are nourished.

The estuarine environment is extremely productive, offering abundant amounts of food in a wide range of sizes. This is one of the advantages of retaining an estuarine phase in the reproduction of some *Macrobrachium* species. Another desirable feature of brackishwater over the freshwater environment is that of a reduction in the osmotic stress imposed upon the larvae, due to the similarity in osmotic pressure of brackishwater and the body fluids. There is another consideration as to why the larvae of some species appear to require brackishwater for successful development. This is the possibility that the larvae may require some nutritional component that can only be obtained in sufficient quantity in the brackish/marine environment.

In their natural environment it is assumed that larval crustaceans initially feed on phytoplankton and as they grow they begin to feed on zooplankton and any food/detritus particle which are of a size that they can catch and hold on to. One of the principal forms of zooplankton fed to decapod larvae in laboratory based trials is the brine shrimp, artemia (*Artemia spp.*). This branchiopod crustacean has many advantages for culture work, so much so that it has become a principle larval feed for many commercially cultured fish and crustacean species. The attraction of using artemia in larval culture is that it has no physical means of defence other than its tolerance of extremely high salinities and an ability to form dormant cysts. These cysts can be easily stored and hatched when needed which means they are a convenient food source in larviculture. In addition, they provide an excellent high protein larval feed food source that is self-suspending and does not leach its nutrients.

2.2.1 Nutritional quality of prey

The increasing use of artemia for rearing the larvae of marine organisms gave rise to the realization that some strains of artemia gave better survival rates than others. It was found that larvae reared on inland strains of artemia (e.g. Great Salt Lake, San Pablo Bay) had low rates of survival (Bookhout & Costlow 1970; Provenzano & Goy 1976; Goy & Costlow 1980; Johns *et al.* 1980). Initially, this difference was attributed to the contamination of some sources with pollutants, especially the Great Salt Lake (GSL) strain from Utah (Bookhout & Costlow 1970). Subsequently, the difference was proposed to be of a biochemical nature. This was due to the fact that good survival of *Palaemon serratus* was obtained by feeding Great Salt Lake strain artemia in conjunction with the unicellular alga *Isochrysis galbana* (Wickins 1972). Work with the larvae of *Macrobrachium rosenbergii* showed that growth and survival was enhanced by culturing the larvae in brackishwater that contained a bloom of phytoplankton. Other workers reported that larval growth was improved by adding unialgal cultures of phytoplankton to the rearing water. This gave rise to the question as to the role of the algae in the rearing tank and whether the larvae were deriving a nutritional benefit from them (Maddox & Manzi 1977). Roberts (1974) reported that some crustacean larvae egested intact diatoms and concluded that the frustule of the diatom might have been unattractive or indigestible, causing the larvae to reject it.

Brick (1974) suggested that the observed improvement in survival of the larvae was a result of the algae removing potentially toxic metabolites from the rearing water (e.g. ammonia, nitrites and nitrates) and adding oxygen to the water. Manzi *et al.* (1977) found that larval *Macrobrachium rosenbergii* had algal cells present in their guts when reared in "greenwater." They also found that more cells were present in later stage larvae. Study of the uptake of algal fatty acids showed that they were not incorporated into the larvae, although the possibility of their immediate utilisation was not ruled out. Joseph (1977) suggested that the beneficial effect of the algae was possibly due to a water soluble leachate from the algae. McConaugha (1985) maintained that decapod larvae derive considerable nutritional benefit from algae, although it would be inadequate as a sole food source. He suggested that, due to the the natural patchiness of plankton distribution, the ability to utilize algae may enable a larva to stay alive in periods of low zooplankton

abundance.

The question as to whether pollutants were the cause of differences between the different artemia strains was resolved by Olney *et al.* (1980). By feeding marine fish larvae with artemia from the Great Salt Lake and also with an Italian marine strain known to be contaminated with DDT, they were able to show that the marine strain gave better results. This pointed to the fact that the poor performance of the inland strains was due to some feature of the inland waters that they inhabited. Fujita *et al.* (1980) suggested that the Great Salt Lake and San Pablo Bay strains were deficient in an essential polyunsaturated fatty acid (PUFA) 20:5 n3 (eicosopentanoic acid). The concentration of 20:5 n3 and 22:6 n3 PUFA is particularly low in non-marine species of algae but high in marine species (Sargent *et al.* 1989). Since the principal food source of artemia is unicellular algae the nutritional composition of those algae will, to some extent, be reflected in the composition of the artemia. This is particularly the case as regards 20:5 n3 and 22:6 n3 fatty acids.

It would therefore seem that low dietary levels of 20:5 n3 and 22:6 n3 PUFA result in poor larval growth and survival in marine decapod species. It also points to the fact that here is a dietary component not readily available in freshwater that is required for growth and development of the larval *Macrobrachium spp.* What is interesting is how do those species which spend their entire life in freshwater obtain this fatty acid as larvae. One possible assumption is that it is invested in the egg by the female at spawning. This is another possible reason as to why fecundity decreases with the breaking of the link with the sea, as the females' ability to gather and store 20:5 n3 fatty acid must be limited in the freshwater environment.

The requirement for 20:5 n3 fatty acid appears to occur towards the later stages of development. In a nutrition trial, the larvae of the crab *Rithropanopeus harrisii* were fed a freshwater strain of artemia for the first half the normal duration of zoeal development, and then switched to a marine strain for the second half. This resulted in high survival and normal development. It was also found that it did not matter whether the marine strain were fed in the first of second half of the rearing period suggesting that the essential PUFA's are highly conserved (Johns *et al.* 1981).

2.2.2 Enrichment of larval feeds

As was shown in the previous section, the nutritional quality of artemia varies depending upon its origin. Methods exist to increase the amount of PUFA in deficient strains. This is desirable because the inland strains of artemia tend to be much cheaper than the marine strains due to their poorer nutritional quality.

If crustacean larvae can take a larger prey item, as in the case of *Macrobrachium rosenbergii,* the freshwater strains of artemia can be successfully used, provided that they are "enriched". "Enrichment" involves holding artemia metanauplii (24 h old at 28°C) in dilute seawater that contains a milk-like emulsion of oil droplets. The oil is usually a marine fish oil which is rich in n3 PUFA. The metanauplii are able to feed by a process of filtration. Their filter-feeding mechanism is only size selective and the small droplets of oil are collected by the artemia. The droplets aggregate in the gut and when this is full, the artemia can be fed to the larvae. There is a problem with enrichment which might have an effect on the culture of larvae. Upon hatching, the naupliar artemia have a yolk sac that is rich in carbohydrate and lipid. Once the nauplius moults to the metanauplius stage there is a rapid decrease in the concentration of carbohydrate, and thus the energy value of the artemia also decreases (Szyper 1989). This loss of carbohydrate may be compensated for by the oil taken up by the artemia .

Artemia hatch as a non-feeding nauplius, the size of which varies depending on the strain. As they do not feed it is difficult to enrich these nauplii, although the enrichment emulsions used may coat the nauplii sufficiently to have some effect. A second problem is that of the increase in size that occurs during the enrichment process. This means that if larvae cannot take a prey size larger than the size of a nauplius it may become deficient in essential PUFA if fed on a freshwater strain as the nauplii cannot be enriched effectively. This may be the case with the larvae of those marine crustaceans which hatch as nauplii and have an extended larval development. These species have little yolk reserves and those which they have are consumed by the time they are large enough to begin to feed on artemia.

The commercial product 'SELCO' (Artemia Systems, Ghent, Belgium) is an enrichment emulsion. It consists of a marine fish oil that is supplemented with extra n3

PUFA. 'SELCO' also contains pigments and vitamins and an extremely strong emulsifying agent. When shaken with seawater, 'SELCO' forms a milky emulsion. The droplets of oil are approximately the same size as algal cells and so they are readily ingested by the artemia.

Another means of enrichment that can be used is a homemade emulsion. It is simple to prepare and is substantially cheaper than the commercial forms. This method of enrichment was designed by Watanabe *et al.* (1982), a recipe for which is given in Appendix II. Fish oil is mixed in a blender with egg yolk and seawater. The resulting emulsion is shaken and added to the artemia enrichment tank. The emulsion can be kept in a refrigerator for several days and so it does not need to be prepared fresh every day, although this would be preferable. The emulsion is added at a concentration of $1cm^3.dm^{-3}$ of seawater in the enrichment tank. The advantage of this second method is that it enables the enrichment of Great Salt Lake artemia to a standard that will give good growth of *Macrobrachium* larvae without having to resort to the more expensive and less readily available SELCO. Another attraction of the Watanabe formula is that egg yolks contain cholesterol, which is essential to adult marine crustaceans. McConaugha (1985) concludes that cholesterol is likely to be essential for larval crustaceans since it is required by the adults. Briggs *et al.* (1988) found no difference in growth between juvenile *Macrobrachium rosenbergii* fed different concentrations of cholesterol in their diet, and concluded that the semi-purified diet contained sufficient cholestrol. The possible requirement for cholesterol or n3 PUFA is another case where the abbreviation of development may require the female prawn to invest extra valuable nutrients in her eggs, as these nutrients might be difficult to obtain in the freshwater environment.

2.2.3 Inert feeds

Most commercial hatcheries do not use artemia as the sole form of nutrition for the larviculture of either *Penaeus spp.* or *Macrobrachium spp.* as this would render the post-larvae produced too expensive. This is particularly the case now that hatcheries are producing such large quantities of larvae. The survival rate in these systems can be very low but the abundance of broodstock from the growout ponds (*Macrobrachium*) and wild broodstock (*Penaeus*) means that this can be tolerated, provided the post-larvae are produced cheaply enough. The feed used in Thailand and Malaysia to supplement the artemia

is called "egg custard". This is a steamed mixture of eggs and a variety of fish products such as, shrimp paste, mussels, cockles, tuna, or any oily fish. The fish products act as an attractant as well as possibly supplying essential oils and amino acids not found in eggs.

Other larval feeds include a gelled egg and squid mixture that is bound using sodium alginate, and ground artemia flake. Egg custard is readily taken by larvae and forms an increasingly large part of the diet as the larvae grow. The egg custard can be dyed to improve its attractiveness, red giving the best response (Ang, unpubl. data). Egg custard does not supply all the requirements of the larvae and artemia are usually fed in the evenings. The use of egg custard and other inert feeds have one major disadvantage and this is their deleterious effect on water quality. Because they are neither alive nor membrane bound, considerable leaching of nutrients occurs. This can cause a problem if overfeeding occurs, or if the water is not changed rapidly enough. Most South-East Asian hatcheries rear shrimp and prawn larvae in greenwater systems, and the algae in these systems will alleviate the problem of poor water quality to some extent. There are frequent outbreaks of disease and mass larval mortalities in the hatchery systems employed in S.E. Asia and the majority of these can be traced back to overfeeding, poor water quality or inadequate nutrition by the use of inert feed. The problem of water quality deterioration is generally overcome by the use of low stocking densities (<30 larvae/litre) and frequent water changes. A problem with the use of inert feeds can arise if the hatchery wishes to stock the tanks at higher densities, reduce water exchange, or both.

2.2.4 Prey/food density

The quantity of food consumed by the larvae in a laboratory or hatchery culture situation is considerable. So much so, that it would be impossible for those larvae to encounter such food densities in their natural habitat (Kon 1979; Paul *et al.* 1979; Anger & Nair 1979). The high levels of food given to the larvae are an attempt to keep them growing at a maximal rate and to discourage cannibalism. That the larvae are able to survive on lower food densities implies that they must be able to withstand periods of starvation, or be able to grow at a reduced rate. There is evidence that larval crustaceans are able to vary the number of stages that they pass through prior to metamorphosis. Thus during periods of low food availability/density there is an increase in the number of

larval stages and the duration of the intermoult period (Broad 1957; Reeve 1969; Knowlton 1974). Food availability is not the only factor affecting the rate of larval development; adverse salinity, temperature and pollution conditions can all retard larval development (Johns 1982).

Thus it would seem that in the ideal culture situation the number and duration of larval stages is at a minimum. This ability to vary the duration of larval life is a sound strategy for survival in the natural environment. By being able to survive a wide range of conditions and, as a result, having a variable time to metamorphosis, the likelihood of larvae encountering the correct conditions for further growth and ultimately metamorphosis is maximised. However, there is the view that the variability in development times is not entirely linked to external factors and that there is an internal/genetic component that affects the rate of development. In the mass rearing of *Macrobrachium rosenbergii* larvae the time to metamorphosis can vary between 18 to 50+ days. It is possible that more synchronous larval development will be achieved as our understanding of the absolute nutritional requirements of the larvae and the optimal environmental rearing conditions increases. The general lack of synchrony in the metamorphosis of the larvae could be due to the larvae waiting to obtain some vital factor in sufficient concentration to allow them to proceed through metamorphosis. This component could be derived from the diet or the environment and could be a trace element, essential amino acid, essential fatty acid or possibly a vitamin. Alternatively, synchronous development could be improved by the animals growing at a maximal rate which is a function of temperature, food density, food quality and stocking density.

2.3 Water quality and rearing environment

2.3.1 Stocking density

A variety of stocking densities have been used over the years (Table I) with a tendency to increase numbers per unit volume. The number of larvae it is feasible to stock in a given volume is dependent upon several factors. The prime limitation on the stocking density is the rate at which water quality deteriorates. The more larvae per litre, the more food needs to be provided and hence the greater the volume of water that needs to

be exchanged. Water exchanges are often limited by pumping capacity, water heating, and by loss of food from the tank. The loss of food is an important consideration since this is often a major part of a hatchery's expenditure. Mesh screens are fitted to the water outflows to prevent loss of food. However, these can become clogged if they are too small or if the flow rate through them is too high. Clogging of the filters results in the suspended food in the rearing tank being sucked onto the filter screen. This effectively results in the loss of food and must be avoided. These factors only become important in intensive clearwater recirculating systems where both stocking densities and water flow rates are high. Static clearwater systems have batch water changes daily. Greenwater systems also have batch water changes, although only on an occasional basis.

Table (I) The various stocking densities reported for the larviculture of *Macrobrachium rosenbergii*.

INITIAL	SURVIVAL	COUNTRY	REFERENCE
53 dm^{-3}	11 dm^{-3}	Hawaii	Fujimura & Okamoto (1972)
13 dm^{-3}	1-2 dm^{-3}	Malaysia	Wickins (1976)
13 dm^{-3}	-	Mauritius	"
24-34 dm^{-3}	7-18 dm^{-3}	Tahiti	"
37-45 dm^{-3}	8-16 dm^{-3}	Britain	"
70-112 dm^{-3}	30-60 dm^{-3}	"	AQUACOP (1977)
60 dm^{-3}	18-42 dm^{-3}	Hawaii	New (1990)
30-50 dm^{-3}	10-30 dm^{-3}	Thailand	New & Singholka (1985)
	100 dm^{-3}	French style	"
300-1000 dm^{-3}	-	Taiwan	Hsieh *et al.* (1989)[a]

[a] The taiwanese method involves dilution through the cycle. Thus the final stocking density is not a function of the initial one, and survival is not given.

The taiwanese method reported by Hsieh *et al.* (1989) involves stocking larvae at very high density initially. This substantially reduces the amount of feed needed in the early stages, since feeding is usually a function of the volume of the rearing tank, not stocking density. As the larvae increase in size they require more food and consequently maintaining them at such high densities would result in the fouling of the water. Thus the tank containing the larvae is gradually filled to reduce the stocking density. A similar method is used in a hatchery in Malaysia (Sunghei Bharu, Malacca), but involves the use of greenwater (Pers. obs.). Water exchanges are minimal as the dilution effect of water addition coupled with the presence of phytoplankton maintains good

water quality.

2.3.2 Light

Larvae tend to be positively phototactic, and this results in their tendency to maintain themselves in the upper part of clearwater tanks. This affects the stocking density locally and can result in cannibalistic interactions, particularly of newly moulted individuals. The method by which the larvae shed their exuvia when moulting seems to involve trapping themselves at the air water interface and then struggling free. Whether the carapace changes its hydrophobicity prior to the moult is not reported, but once moulted, the exuviae form a scum on the surface of the rearing tank. This is particularly evident as the larvae increase in size. A concentration of larvae at the surface of the tank is not a problem if they are all moulting. However, if intermoult individuals are present this could substantially increase mortality due to cannibalism.

Larvae in clearwater systems are susceptible to the effects of sunlight, the 'sun-cancer' effect (Fujimura & Okamoto 1970). Clearwater hatcheries are often housed indoors and outdoor tanks are 90% covered to prevent this (New 1990). In greenwater systems, the phytoplankton attenuate the sunlight and provide protection for the larvae, although tanks are usually shaded.

New (1990) suggests that the spectral quality of the light supplied to the larvae should resemble natural sunlight as closely as possible. The colour of the larval rearing tanks might be important as New & Singholka (1985) suggest that dark coloured tanks give better results. However, they also state that "not all successful hatchery operators would agree with this statement". The larvae of *Macrobrachium rosenbergii* have well developed eyes which are present upon hatching. It is not unreasonable to suppose that they catch their prey visually. The larvae do not actively hunt their prey but wait for it to pass them whereupon they seize it. In clearwater tanks with a unidirectional source of light the larvae can be seen to orient themselves around the walls of a dark tank. They swim upside down and presumably look upwards towards the light. It might be that the larvae flick up to catch any object that passes through the field of light. Light entering water is refracted and a single point light source (e.g. the sun) spreads its rays outwards. This has the effect of silhouetting any object shallower than the observer. Thus a possible explanation for the success of dark tanks is that it allows more efficient capture of prey

items as the light will tend to be unidirectional and not reflected off light surfaces.

There has been work performed upon different colourations of egg custard (the standard supplemental feed for larval rearing). Egg custard is normally a yellow colour but red was found to be the most effective colour. It may be that the contrast, and therefore the 'catchability' was greater for the red feed (Ang, unpublished data).

2.3.3 Temperature

Work performed to attempt to establish the optimal rearing temperatures and salinities for the larvae of *Macrobrachium rosenbergii* has yielded different results (Table II). Gomez-Diaz (1987) attributed this variation to degree of acclimation, phenotypic variability or differences in experimental methodology. Temperature is a good example of a parameter that is difficult to find an optimum for. If an animal is poikilothermic, then its metabolic rate should increase with temperature. This in turn should be seen as an increase in growth rate. However, the quantity and quality of food supplied to the growing prawn will also have an effect upon the rate at which it is able to grow. If the temperature is high and the food supplied to the animal is insufficient or of inadequate nutritional quality a different growth rate will be obtained than if the nutrition was optimal. An inadequate supply of food to fast growing larvae could result in elevated rates of cannibalism and a decrease in survival. This would then suggest that the optimal rearing temperature should be lower. The cannibalistic nature of *Macrobrachium rosenbergii* larvae and postlarvae means that survival rate is strongly linked to both feeding and stocking rates. Artemia are generally the preferred feed for larvae in these rearing type experiments, but, as has been shown in the section on larval nutrition (section 2.2.1) these can be of variable quality. If the food item provided is unsuitable the problem of larval cannibalism can arise. This can occur particularly when the larvae moult, as for some time after moulting they are quite torpid. In undisturbed water they tend to sink to the bottom. They can be seen in any sample of larvae from a rearing tank and in this condition it is not unusual to find other larvae feeding upon them, especially if there is inadequate food present.

The time to metamorphosis gives a good indication of the growth rate the larvae may achieve, and a combination of survival and time to metamorphosis enables the

determination of suitable values for rearing temperatures (Table II). New (1990) gives a range of 26-31°C for larval rearing. Hsieh *et al.* (1989) report that some Taiwanese hatcheries rear larvae at temperatures between 30-33°C.

Table (II) Rearing temperatures and lethal limits for the larvae of *Macrobrachium rosenbergii*

Temperature (°C)	Comments	Reference
28-30	optimum,	Sandifer & Smith (1985)
26-31	range,	Sandifer & Smith (1985)
26-31	range,	New (1990)
t>35	retarded development	"
t<24	or mortality,	"
30-33	Taiwanese hatcheries	Hsieh *et al.* (1989)
26-32	range, 8 °/oo	Uno & Yagi (1980)
26-35	range,14 °/oo, 20 °/oo	"
29-31	range, 27 °/oo	"
29-35	range, 33 °/oo	"

The work by Gomez-Diaz (1987) on the effect of different incubation temperatures upon the survival and rate of development of *Macrobrachium rosenbergii* larvae, has yielded some very interesting results. It was found that when gravid females were incubated at low (25°C) and high (31°C) temperatures the resulting larvae had a wider range of temperature and salinity tolerance than those incubated at an intermediate temperature (29°C). The development time to metamorphosis was faster in the high temperature treatments, but only if the gravid female had been incubated at low (25°C) temperature. It is possible that these effects might be due to the utilisation of larval reserves. Larvae reared at 27-28°C can survive for five days without being fed (Ling 1969a). This is due to the larval yolk reserve. This reserve might be conserved if the animal feeds well after hatching. However, if it is exhausted by high temperature embryonic development, then the loss of that reserve might slow down growth post hatch. The stage I larvae do not feed and rely on their yolk reserve to sustain them through the first moult. The yolk reserve may also allow the survival of the larvae in periods of low food abundance. If this reserve is low then the larvae will be more susceptible to

underfeeding.

2.3.4 Salinity

The larvae of *Macrobrachium petersii* osmoregulate over quite a wide range in salinity (Read 1984). This range is 0-35 °/ooS in stage I larvae and postlarvae. Intermediate larval stages showed a reduced capacity to regulate their blood osmolarity below approximately 7 °/ooS, and above approximately 30 °/ooS. The lifestyle of this prawn is similar to that of *Macrobrachium rosenbergii*, and the salinity tolerance values correspond approximately to those found in the larvae of *Macrobrachium rosenbergii* (Ling & Merican 1961). Thus it could be assumed that the osmoregulatory strategy of the two species is the same.

Table (III) Salinities used in the larval rearing of *Macrobrachium rosenbergii*

Salinity °/oo	Comments	Reference
10-30	tolerance range	Ling & Merican (1961)
15-20	optimal rearing	Ling & Merican (1961)
7-8	tolerance range	Sandifer & Smith (1985)
6	some stages	Sandifer & Smith (1985)
10-15	optimal rearing	New (1990)
12	optimal rearing	New & Singholka (1985)
15	-	Nair *et al.* (1977)

It has been suggested that the least energy is expended by a marine animal if it is maintained in an isosmotic medium, due to the reduced energy expenditure on osmoregulation (Canagaratnam 1959). This concept has been considered with respect to adult *Macrobrachium rosenbergii* by Singh (1980), who concluded that it did not apply. This was based on the findings of Sandifer & Smith (1975) that the best growth rates were achieved at a salinity of 2 °/oo. This is probably due to the physiological adaptions of the adults to life in freshwater. However, larval *Macrobrachium rosenbergii* (and *Macrobrachium petersii*) live in brackishwater, and thus the isosmotic concept may hold true in this case. Subramian & Krishnamurthy (1986) reared *Penaeus monodon* and *Penaeus indicus* in a variety of saline waters (13, 23, 33 °/ooS) and found the best

growth at the isosmotic salinity of 23 °/oo. The isosmotic point for *Macrobrachium petersii* is approximately 17.5 °/ooS and is higher than the rearing optima listed in Table III. The optimal salinity for larval rearing seems to lie between 12-15 °/ooS.

2.3.5 Ammonia, nitrite and nitrate

One of the major problems that beset early efforts to rear *Macrobrachium rosenbergii* larvae on a large scale, was the rapid deterioration of water quality (Fujimura & Okamoto 1970; Dugan *et al.* 1975; AQUACOP 1977). The cause of this deterioration was a combination of nitrogenous excretory products from the larvae and artemia, dissolved organic material from the feed and crashed algal blooms. The greenwater system attempts to keep a balance between all the components of the culture tank: larvae, phytoplankton, zooplankton, bacteria and feed. However, problems with water quality arise when the equilibrium between these components is upset. The clearwater system, whilst more controllable is still susceptible to high organic loadings. These occur principally due to the higher stocking densities employed. This results in a greater amount of feed being added to the rearing tank, and the increased likeliehood of fouling the water, even with the frequent water exchanges employed.

The use of recirculation systems is an ideal method for the maintenance of good water quality and has gained favour with the users of French intensive systems. This probably stems from the early experiences of AQUACOP (*Centre Oceanologique du Pacifique,* Tahiti). Where recirculation systems are used, it is important that regular water quality checks, in the form of nitrite and ammonia tests, are made on culture tanks. This allows the efficacy of the biological filter to be assessed, and potential filter 'crashes' to be avoided. A 'crash' in a biological filter occurs when the equilibrium between nitrification and denitrification becomes disturbed. The result is that instead of a net nitrification process there is net denitrification (Spotte 1979). This gives rise to very high concentrations of ammonia and nitrite in the culture system which can be lethal to the larvae (Tables IV,V).

The LC_{50} values for ammonia toxicity presented in Table IV are informative as they provide an indication as to the concentrations which are intolerable in

larval culture. In a recirculating system, and indeed in any aquaculture system, the lowest possible ammonia concentration is the most desirable. Wickins (1976), studying the toxicity of nitrite, nitrate and ammonia to adult warmwater shrimp and prawns, found *Macrobrachium rosenbergii* to be less tolerant than *Penaeus monodon*. He also gives approximate EC_1 and EC_2 values for unionised ammonia of 0.09 and 0.11 mg NH_3-N.dm^{-3} respectively. The EC_1 and EC_2 values are those concentrations of unionised ammonia that reduce the growth of an animal by 1% and 2% of the control. His data for *Macrobrachium rosenbergii* indicate that a concentration above 0.1 mg NH_3-N.dm^{-3} (unionised ammonia) reduces growth by 60-70% of that of the controls. This gives a value of 1.6 mg.dm^{-3} total ammonia for the temperature and salinity conditions encountered in larval culture (12°/oo, 28°C, pH 8.0). These figures are thus a better indication of the ammonia values to be tolerated in a larval rearing system.

Table (IV) Toxicity of Ammonia to *Macrobrachium rosenbergii* larvae at different pH levels - Adapted from Armstrong *et al.* (1978)

pH	total Ammonia	NH_3	NH_4^+	percent unionised
	24-hour LC_{50} (mg.dm^{-3})			
6.83	200	0.66	199.34	0.33
7.60	115	2.10	112.90	1.83
8.34	37	3.58	33.42	9.68
	144-hour LC_{50} (mg.dm^{-3})			
6.83	80	0.26	79.74	0.33
7.60	44	0.80	43.20	1.82
8.34	14	1.35	12.65	9.64

The presence of ammonia in the culture water is an indication that it is fouled. Poor water quality in the form of high ammonia and nitrite concentrations is an environmental condition that a marine/brackish organism would not encounter in its natural habitat. This being so, elevated concentrations of these nitrogenous metabolites will present some form of stress to the animal being cultured and may impair its

performance.

New (1990) gives a figure of 0.1 mg.dm^{-3} NO_2-N for a safe level for larviculture of *Macrobrachium rosenbergii*. This is very low and it is very difficult to maintain static water larval rearing tanks at this level. Greenwater systems can achieve this at low stocking densities, but clearwater systems would require some degree of biological filtration to maintain such a low concentration. Armstrong *et al.* (1976) report that nitrite concentrations of 1.8 mg NO_2-N.dm^{-3} resulted in reduced growth in *Macrobrachium rosenbergii* larvae.

Table(V) Maximum nitrite concentrations that resulted in no mortalities of *Macrobrachium rosenbergii* larvae, over three exposure periods (Armstrong *et al.* 1976).

EXPOSURE TIME		
24 h	96 h	168 h
(mg NO_2-N.dm^{-3})		
9.7	3.3	1.0
9.7	1.0	-
9.7	1.8	1.8

Nitrate is seldom a problem in aquaculture systems, as the rate of water exchange usually keeps the level low. New (1990) gives a concentration of 20 mg NO_3-N.dm^{-3} as a tolerable concentration of nitrate, although this figure is unreferenced. Spotte (1979) gives the same figure as a safe level, and reports that toxicity of nitrate to salmon in seawater was 1.14-1.41 times less than the toxicity in freshwater. There are no data concerning the toxicity of nitrate to larval prawns, although Wickins (1976) found LC_{50} and EC_{50} values of 160 and 175 mg NO_3-N.dm^{-3} respectively for adult *Macrobrachium rosenbergii* maintained at a salinity of 3 $^{o}/_{oo}$.

2.4 Conclusion

The larvae of *Macrobrachium rosenbergii* appear to tolerate a wide range of environmental conditions. This is understandable since this species belongs to the Palaemonidae which are very well adapted to life in brackishwater, an environment characterised by fluctuations in the chemical and physical environment. Whilst the larvae are tolerant of environmental variation, this does not mean that growth and development are unaffected. Thus, in order to study the growth and development of this species it is necessary to optimise the culture conditions. This optimisation involves the control of the chemical and physical conditions of the rearing environment, and the provision of adequate nutrition. This is especially important with respect to the study of factors that might only have an effect over a long term. If there is high larval mortality due to poor water quality, nutrition or cannibalism then enhancement or retardation of growth and development might be masked. The larval culture conditions chosen are described in section 3.2.

CHAPTER 3 - General methods and materials.

3.1 Rearing adult *Macrobrachium rosenbergii*

3.1.1 Maintenance of adults/broodstock

Three recirculating systems were employed in the maintenance of the prawns. These were; 1) individual broodstock tank system, 2) communal broodstock and postlarval ongrowing system, 3) individual experimental tank system.

System 1 contained large male and female broodstock prawns to provide larvae for experimental work. The tanks used in this system were 0.19m^2 ground area, and they were filled to a depth of 18cm. Since these prawns were held individually, a daily record of moulting was kept and matings were managed (section 3.1.3).

System 2 consisted of eight 1m^2 fibreglass tanks (filled to 25 cm depth) four were translucent and four coloured grey. In this system, three tanks contained a large bull male (blue clawed), and approximately eight females held communally. These tanks provided a steady supply of gravid females, although they were smaller than the females in system 1. The smaller size of these females meant that they produced fewer larvae. The other tanks in this system were used for the ongrowing of postlarvae produced in larval rearing trials. These would then be introduced to the broodstock tanks as they reached an appropriate size.

System 3 was the largest of the three systems and consisted of 40 x 0.10 m^2, and 36 x 0.21m^2 white polypropylene tanks. Water depth in these tanks was approximately 20 cm. Adult prawns were held individually and used for experimental work. Prawns were checked daily and moulting was recorded.

Broodstock prawns were originally obtained from Thailand, and bred and maintained at the Institute of Aquaculture, Stirling. Adult prawns were maintained in the freshwater recirculating systems listed above. These systems were held at a temperature

of 27°C, and tanks were individually aerated. There was a 12 : 12 hour light : dark regime, provided by white fluorescent striplights. Green diffusers were used to decrease the light intensity in system 3.

Water hardness and pH were checked weekly in all recirculating systems. Attempts were made to maintain water hardness between 40-150 ppm $CaCO_3$. These were the values recommended by New & Singholka (1985). Due to the soft water in the Stirling area, calcareous filtrant in the form of crushed cockle shell was included in the biological filters of the recirculating systems. This caused a gradual increase in water hardness with time. Replacement of water in the system with soft tap water reduced this, and thus water exchange was the method for controlling high water hardness. Water exchange occurred naturally to some extent as a result of cleaning tanks and leakage from the system, necessitating topping up with tap water. Water hardness in excess of 150 ppm $CaCO_3$ did occur occasionally even with substantial water exchanges. The result of this was the development of black lesions on the carapaces of prawns. Usually these would be lost at the next moult, provided the water hardness was reduced. In some instances prawns would die in the act of moulting.

Normal pH values in the systems were approximately 7.0. Occasionally the pH of the systems decreased and this was attributed to the loss of buffering capacity caused by insufficient cockle shell, or the fouling of the filters (Spotte 1979). On the occasions when this occurred the shell would be washed and/or replaced. If this occurred in conjunction with high hardness, $NaHCO_3$ would be added to improve the buffering capacity of the water. This increased pH without adding calcium or magnesium to the water.

The biological filter of system 3 was cleaned annually due to the build up of organic matter, mainly bacterial floc. This took place over several days to avoid major disturbance to the denitrification processes.

3.1.2 Adult, juvenile and postlarval prawn nutrition

Prawns were fed a mixture of feeds depending upon their size and the type of system in which they were maintained. Postlarvae were fed on crushed flake fish food after transfer from larval culture tanks. They were subsequently weaned onto an adult

diet. The adult broodstock diet consisted of a formulated pellet, chopped fish, liver, fresh mussel, green beans and spinach. Green vegetables were included in the diet to provide vitamin C, since a deficiency seems to cause moult death syndrome (M. Briggs, pers. comm. 1988). Moult death syndrome is the mortality of prawns in the act of moulting. Typically, a prawn was found dead trapped in the exuvium which it was trying to shed. In less severe cases the prawns were able to shed the exuvia but would possess deformed appendages. This occurred occasionally at the beginning of this work in system 3, but ceased once the diet included a formulated pellet (containing vitamin C, vitamin mix) and green beans supplied twice a week. Indeed, the prawns in this system were maintained entirely on the formulated diet and green beans for extended periods with no ill effects. Feeding was on an *ad libitum* basis. Prawns were not overfed since this caused fouling of the water. The formulation for the pellet fed to the adult prawns is given in Appendix 1.

3.1.3 Breeding

Prawns held individually in system 1 were used for managed breeding. This involved the introduction of a blue clawed (bull) male into the tank of a female that had just moulted. The two prawns were left undisturbed for approximately four hours, after which the male was removed. Extrusion of the eggs occurred on the same day, and were seen as a yellow/orange mass on the pleopods of the female. Egg extrusion occurred whether the female was fertilised or not, but does seem to be dependent upon the quality of the diet. The state of 'ripeness' of the ovary is easily seen in this species. It appears as an orange/yellow mass in the dorsal cephalothorax. If the ovary appears large and very orange/yellow this is a good indication that the female is ready to spawn at the next moult.

Whether the eggs have been successfully fertilised is not known until several days after mating. If the female has not been successfully mated the eggs begin to detach and will be eaten by the female. It is assumed that this is the best method for conserving the valuable nutrients invested in the eggs. Fertilised eggs remain attached to the pleopods for approximately 20 days (at 27°C) before hatching. Approximately fourteen days after fertilisation the eggs begin to darken, this is due to the development of the eyes in the embryos, and was generally used as the indicator for when to introduce the female to brackishwater for larval hatching. Hatching usually occurred overnight approximately six days after the eggs turned grey, and the spent female would be removed

the following morning. Some workers report that larvae hatch in two batches over two days (Wickins & Beard 1974; Gomez-Diaz & Ohno 1986). Gomez-Diaz & Ohno (1986) suggest this occurs if the female is held at suboptimal temperatures (≤25°C). However, Wickins & Beard (1974) found that 41% of females spawned over two days when held at 28 ± 1°C. This was very rarely observed in this work with spawning temperatures of ≈ 28°C.

3.2 Rearing of larval *Macrobrachium rosenbergii*

The larval culture system was originally designed to allow several aspects of larval culture to be studied. These aspects were all concerned with the functioning of a recirculating larval rearing hatchery that uses artificial seawater. These were:

1) Whether the artificial seawater formulated is suitable for the successful production of postlarvae.

2) Whether the ionic composition of artificial seawater could be simplified by establishing if some ions are inessential for larval growth.

3) Whether the artificial seawater could be used for a larval rearing cycle without being changed.

To attempt to answer these questions, a recirculating larval culture system was designed that incorporated biological filtration, and that needed no water changes over the duration of a larval cycle. By not changing water over the course of a larval rearing cycle it was hoped that any significant changes in the concentrations of the major constituents in the seawater could be detected.

3.2.1 Design of larval culture tank (Fig.1)

The larval rearing vessels were 70 litre black polypropylene food hoppers. The conical shape of the vessels was to ensure that the aeration of the tank caused the suspension of larvae and artemia. In preliminary rearing trials, white conical vessels were used. It was found that the larvae did not feed well and they soon succumbed to

infections of filamentous bacteria, *Zoothamnion* and ectocommensal protozoa. These indicated possible water quality problems, but even with 100% daily water exchange, the larvae still died. This problem was resolved by the use of black tanks and the conclusion drawn was that the incidental light in the white tanks had been too great. Whether the interference with feeding behaviour was due to stress from high light levels, or the lack of directionality of the light has not been resolved.

3.2.2 Biological filters

Brackishwater (12°/oo) was recirculated in the larval rearing vessels by an 'Eheim' biological filter. This is a sealed container that contains filter media such as gravel and shell, which act as substrata upon which bacteria can develop. The bacteria develop naturally in the filter in response to the various organic compounds that appear in the water as a result of the culture process and break down organic compounds in the water, e.g. nitrite, ammonia, dissolved organic carbon compounds (Spotte 1979). It was essential that these bacteria were present prior to the introduction of the larvae, otherwise there would have been a period of poor water quality as the filter settled.

The easiest method for settlement of a biological filter for larval rearing was found to be the introduction of a gravid female prawn whose eggs had just turned grey. The tank would contain 12 °/oo seawater. She would be lightly fed during her stay in the tank (approximately 6-10 days, at 27°C), and removed once the larvae hatched. This method of filter settlement was found to be completely reliable, and nitrite in the early stages of larval culture was always at undetectable levels.

Settlement of several filters for a rearing experiment was achieved by the introduction of low concentrations of artemia several days prior to larval introduction. Since the artemia were not fed they gradually died off thereby providing organic material for the filters. The small size of the newly hatched larvae and their comparatively low feeding rate probably helps settle the filters as well. Spotte (1979) cites Kawai *et al.* (1964) as to the times for development of maximum densities of denitrifying bacteria in a warm seawater aquarium. These were 4 weeks for nitrite formers and 8 weeks for nitrate formers. This seems to be slower than the biological filters used in the present trials.

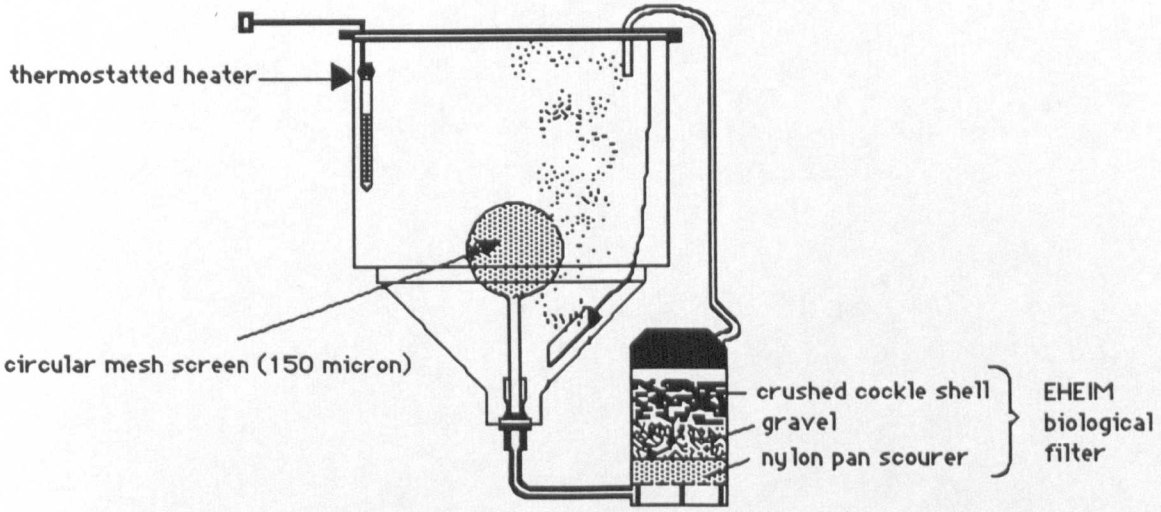


Fig.1 70 dm^3 larval rearing tank

The biological filter and media were always thoroughly washed between rearing trials. This was to prevent contamination of the various artificial seawater media used, and also to avoid filter 'crashes'. The build up of organic material in the filter also gradually reduced the flow rate but washing the filter restored this to normal. The use of fresh filters for each larval rearing cycle ensured that the filter was always in equilibrium with the organic loading in the culture tank.

Each filter contained the same volumes of each type of filter media. These were, crushed cockle shell 30% (1.2 dm^3), coarse gravel (non calcareous) 30% (1.2 dm^3) and a plastic pan scourer 16 % (0.64 dm^3). The filter media provided a large surface area for bacterial settlement, and acted as a mechanical filter. The crushed shell was included to aid the buffering capacity of the seawater, although Bower *et al.* (1981) suggest that this will not maintain the pH above ≈7.5. Plastic spacers were used at the top and bottom of the filter to improve flow characteristics. Total filter volume was 4 dm^3. A magnetically driven impeller housed in the top of the filter circulated the water at a rate of approximately 5.5 $dm^3.min^{-1}$.

A screen was employed to prevent the loss of larvae and artemia into the filter from the culture tank. This was a double sided circular screen with a mesh size of 150 μm (total area = 480 cm^2). This size mesh is just small enough to prevent artemia passing through. This mesh was washed daily since it gradually became clogged with dead or dying artemia. If the screen was not thoroughly washed a bacterial slime accumulated within the meshes. This lowered the effective mesh size and caused a more rapid clogging of the screen. The use of detergent or drying prevented this. Clogging of the screen could result in lowered flow rates through the filter and an increase in the nitrite concentration in the water.

Daily removal of uneaten artemia in experimental trials was performed using a wide mesh screen (350 or 500 μm). These screens prevented the passage of larvae but allowed artemia through. Water and artemia were siphoned out of the tanks daily via the wide mesh screen, the artemia were trapped in a 150 μm screen, and the water returned to the larval culture vessel. The smaller of the two screens was used when the larvae were less than five days old.

3.2.3 Water quality

The nitrite concentration of the rearing water was checked daily in the larval culture tanks by the use of a 'TETRA' NO_2-N test kit. This uses the reaction between sufanilamide and N-(l-napthyl)-ethylenediamine (NED) that yields a coloured product upon reaction with nitrite (Strickland & Parsons 1972). Depending upon the concentration of nitrite in the rearing water the colour range is between light yellow and dark red. An approximate nitrite concentration can be derived by comparison with a colour chart. Values below 0.25 mg NO_2-N/dm^3 (light yellow-light orange) were considered to be acceptable and were rarely exceeded. During routine larval rearing, elevated nitrite concentrations would be corrected by water exchange and reduced feeding. A reduction in the feeding rate was undesirable as it gave rise to increased rates of cannibalism. This was easily seen by the number of larvae found grasping other larvae, when a sample was taken in a beaker. If high nitrite values were detected the ammonia concentration of the rearing water would be checked. This gave an indication as to whether the biological filter was functioning at all.

Generally, the biological filters maintained the concentrations of nitrite and ammonia within the safe limits recommended, and problems only occurred when the larval stocking density was very high. A more frequently encountered problem was the stopping of the recirculation pumps. This occurred during power failures and occasional air locks in the filter. The loss of flow caused a rapid stagnation of the filter and build up of ammonia and nitrite in the standing water of the filter. This would then be passed into the rearing tank when the flow resumed. An additional problem was the build up of nitrogenous waste in the larval tank due to the lack of recirculation. The filter would restabilise quite quickly after these occurrences provided substantial water exchanges were performed.

Water exchange was not possible in those experiments which attempted to demonstrate that the same water could be used for a full larval culture cycle. If nitrite concentrations increased significantly, feeding would be reduced in an attempt to reduce the organic loading to the water and the filters.

Water temperature in the larval rearing tank was maintained by aquarium heaters set to 28°C. The heaters maintained the tank water consistently at this

temperature ± 1°C. Occasional power failures caused water temperatures to fall to ambient air temperature which was approximately 23°C. This did not seem to have an appreciable effect on the larvae over the time they were subjected to the low temperature. Temperature was checked daily in experimental tanks using a hand held thermometer.

Aeration was provided by a high volume blower (Crompton Gryphon, class E, Huddersfield, England) and delivered via airstone diffusers. Air blower failure causing loss of aeration caused considerable stress to the larvae, especially if coupled with a loss of recirculation. In some instances 100% mortality of the larvae occurred. The level of aeration was not monitored and was set by eye. Vigorous aeration was considered undesirable because it caused undue turbulence in the tank which resulted in damage to the larvae. This was most often seen as bent, or melanised rostrums. Vigorous aeration was also thought to interfere with feeding. Thus the level of aeration used was sufficient to suspend the artemia in the tank and visibly circulate the water, without giving it the appearance of 'boiling'. Measurement of the dissolved oxygen concentration of the water at this level of aeration showed it to be fully saturated.

The salinity of the rearing water was 12 °/oo and was checked using a hand held refractometer accurate to ±1 °/oo. The salinity increased during the course of a rearing cycle due to evaporation, and would be periodically adjusted to 12 °/oo using deionised water.

3.2.4 Larval nutrition

Artemia was the sole food source used throughout the larval rearing cycle. It was decided to use only artemia for two reasons. Since artemia is a living organism it does not disintegrate in the rearing water as do inert feeds and it is therefore easier to maintain good water quality. Artemia also suspends itself in the water column better than inert feeds. This is due to the positive phototaxis of the artemia which keeps them swimming towards the top of the tank. Inert feeds require quite strong aeration to prevent them settling out onto the bottom of the tanks which accelerates their degradation. Artemia is known to be able to support crustacean larval growth past metamorphosis and is generally considered to be nutritionally superior to inert feeds.

In preliminary experiments there was a concern that the feed would introduce unwanted ions and obscure the results of the experiment. Yet feed must be introduced to the system to maintain the larvae. It was an assumption of this work that the feed contained trace elements, and that these would be available to the larvae in a commercial culture situation. Therefore their essentiality, whilst indeterminable, would not be a constraint on the potential use of an artificial seawater.

3.2.5 Larval feeding

The larval yolk reserve at hatching is sufficient to support the larvae for 4-5 days in either freshwater or 12 °/oo seawater. Whilst the stage I larva does not moult if hatched in freshwater, it does so in seawater and its yolk reserve is sufficient to sustain it until it moults to stage III in some cases. However, the larvae were always fed from 24 hours after hatching.

Larvae were fed once or twice daily and always to excess if possible. This was achieved by introducing sufficient artemia to the larval culture tank to ensure there were still some left at the following feed. This was not always possible as blocking of the filter screens tended to remove the artemia from suspension. If the screens were cleaned thoroughly this problem was minimised. Although a single feed is not desirable as far as maintenance of a steady feed density is concerned, it was the only method possible that allowed daily removal and replacement of artemia in the morning. The artemia were removed daily in some experimental rearing trials in an effort to minimise the leaching of ions into the rearing water. With a shift in feeding time to the late afternoon it was possible to institute an intermediate morning feed to boost the artemia density in heavily stocked rearing tanks. Generally, the larval density was such that the quantities of artemia provided were sufficient to leave some left by the next feed. By feeding to satiation it was hoped that this would negate to some extent the differences in stocking densities between treatments.

The larvae were checked daily in all treatments to establish whether they were feeding, and to determine their nutritional state. It was unusual to find larvae grasping and consuming artemia, although this is stated as being the method by which satisfactory feeding can be determined. Similarly, observation of the hind gut was not a

good method by which to establish if the larvae have been feeding. It appeared that the contents of the hind gut were rapidly voided once they entered it. The faecal strands that can be observed in penaeid larvae which have been fed upon artemia and algae were never seen (pers. obs.). Observation of the larvae under a compound microscope (x40) to reveal the degree of fullness of the stomach and digestive gland was found to be the best method by which nutritional state could be established. The use of enriched artemia resulted in the accumulation of large oil droplets in the larvae. These were clearly visible and the extent to which these filled the cephalothoracic cavity was taken as the degree of 'fullness' of the larvae. Larvae that were underfed, or which were not feeding, had no such visible reserves or the orange colour associated with artemia consumption; their cephalothoracic cavity would appear translucent and empty.

3.2.6 Artemia hatching and enrichment

Only Great Salt Lake strain artemia were used for this work, although the brands were different (Argent, Artemia Systems, San Fransisco Bay Co.). Since it has been postulated that the fatty acid profile of this strain is inferior to marine strains of artemia (see larval nutrition 2.2.1) the artemia were enriched using emulsified fish oil compounds ('SELCO', 'SUPER SELCO', homemade emulsions 1 and 2). The different enrichment compounds were used due to problems encountered obtaining 'SELCO.' Enrichment of the artemia had the added advantage of giving a bigger artemia which may have been easier to catch. The degree of enrichment could be assessed by the quantity of oil droplets observed in the gut of the artemia after a fixed period of enrichment. The recommended level for SELCO is 0.3 $g.dm^{-3}$ of enrichment water (Artemia Systems Ltd.). The same level was chosen for the homemade formulation No.2. This may have caused over-enrichment as this formulation was far more viscous than SELCO, implying a higher oil content. The formulations for the homemade enrichment compounds are given in Appendix II.

Artemia cysts were hatched overnight in ≈12°/ooS seawater and ≈28°C. Strong aeration was provided by a straight, 1 cm bore PVC pipe. This was found to be superior to the use of airstones as the 'bumping' effect of the air prevented the loss of cysts onto the walls of the tank, where they would not hatch. Strong light was provided by a fluorescent striplight suspended over the hatching tanks. This gave good hatching after 24

hours. Hatching began to occur after ≈18 hours incubation.

Separation of the hatched artemia from the discarded and unhatched cysts was achieved by removal of the air pipe and covering the hatching tank with a black lid. The tank would be left like this for approximately five minutes and then the artemia would be siphoned from the bottom of the tank where they had settled. The artemia were trapped on a 150 µm mesh and transferred to the enrichment tank. This had the same conditions as the hatching tank, but also contained the enrichment compound being used at the time. Artemia would be left for 24 hours in the enrichment tank before harvesting. The artemia were washed briefly in freshwater prior to being fed to the larval tanks. In experimental treatments they were washed in deionised water. Washing was performed to remove any enrichment compound from the outside of the artemia and also to rinse off the seawater they were maintained in. Some seawater was introduced due to the gut content, but this was not deemed feasible to remove. Artemia hatching and enrichment could have been performed in artificial seawater stock solution (section 4.5), and this would have prevented any seawater access to the experimental tanks. However, the volumes of artificial seawater this would have involved would have rendered it impracticable.

The artemia hatching and enrichment tanks were thoroughly cleaned after use. It was found that if this was not done a build up of bacterial/organic slime occurred. This reduced the hatching of the artemia and was considered to be a disease risk to the larvae.

3.2.7 Estimation of larval numbers

Attempts were made to estimate larval numbers by subsampling in the rearing tanks. However, it was found that, due to their tendency to migrate towards light and preference for maintaining a position against a surface, the larvae were very unevenly distributed in the rearing tank. Increasing the level of aeration improved the distribution of the larvae in the tank, but insufficiently to give results that were closely related to numbers derived by total counts. Thus, the method of evaluating larval numbers was to collect all the larvae in a small volume of water and count them individually. This was extremely time consuming and resulted in infrequent determinations of numbers.

3.2.8 Postlarval separation

Postlarval *Macrobrachium rosenbergii* metamorphose asynchronously, unlike the larvae of penaeid shrimp which metamorphose synchronously (pers obs.). As a result, it was necessary to remove them as quickly as possible from the larval culture vessel. This is because their benthic habit does not bring them readily into contact with the artemia supplied as feed. This can give rise to cannibalism in the rearing tank. Separation is difficult if performed manually as the postlarvae are very agile and not easy to capture. Larvae are easier to remove but, if they form the bulk of the animals in a rearing tank, this is also extremely time consuming. A postlarval separator for use in commercial hatcheries has been reported (Martinez-Palacios *et al* .1985) which relies on the positive rheotaxis of the postlarvae. However, the system operates slowly and requires a large static water rearing tank to be effective. This method gives postlarval separations of up to 82% with *Macrobrachium rosenbergii* over a 24 hour period. In commercial hatcheries it is usual practice to leave larvae and postlarvae together until 90% metamorphosis is judged to have occurred (New & Singholka 1985). The brackishwater in the rearing tank is gradually freshened to acclimate the postlarvae before transferrance to the nursery or growout ponds.

To satisfy the requirement for postlarval separation in rearing experiments, a device was designed to actively separate the larvae from the postlarvae. If this could be achieved crudely then individual larvae and postlarvae could then be separated by hand. The design of the separator was a flat bottomed, circular plastic container (Fig. 2). Water entered the bottom of the tank so as to give a circular, vortical motion, and left the tank via a centrally placed standpipe. Water circulation was achieved by an empty 'Eheim' powerfilter which was screened to prevent larvae entering the pump mechanism. The swirling motion of the water caused the postlarvae to settle to the bottom facing the flow of the entering water. The larvae being more planktonic and with their less well developed swimming ability were carried towards the central drain pipe. The vortex created by the water entering the drain pipe was sufficient to carry the larvae down the pipe. Postlarvae could generally swim away from the pipe and not get caught. Moulting and newly moulted larvae tended to settle on the bottom and were not separated out, and some postlarve were attracted to the flow around the standpipe and were caught by the vortex. Results and discussion of the efficacy of the separator are given in Appendix III.

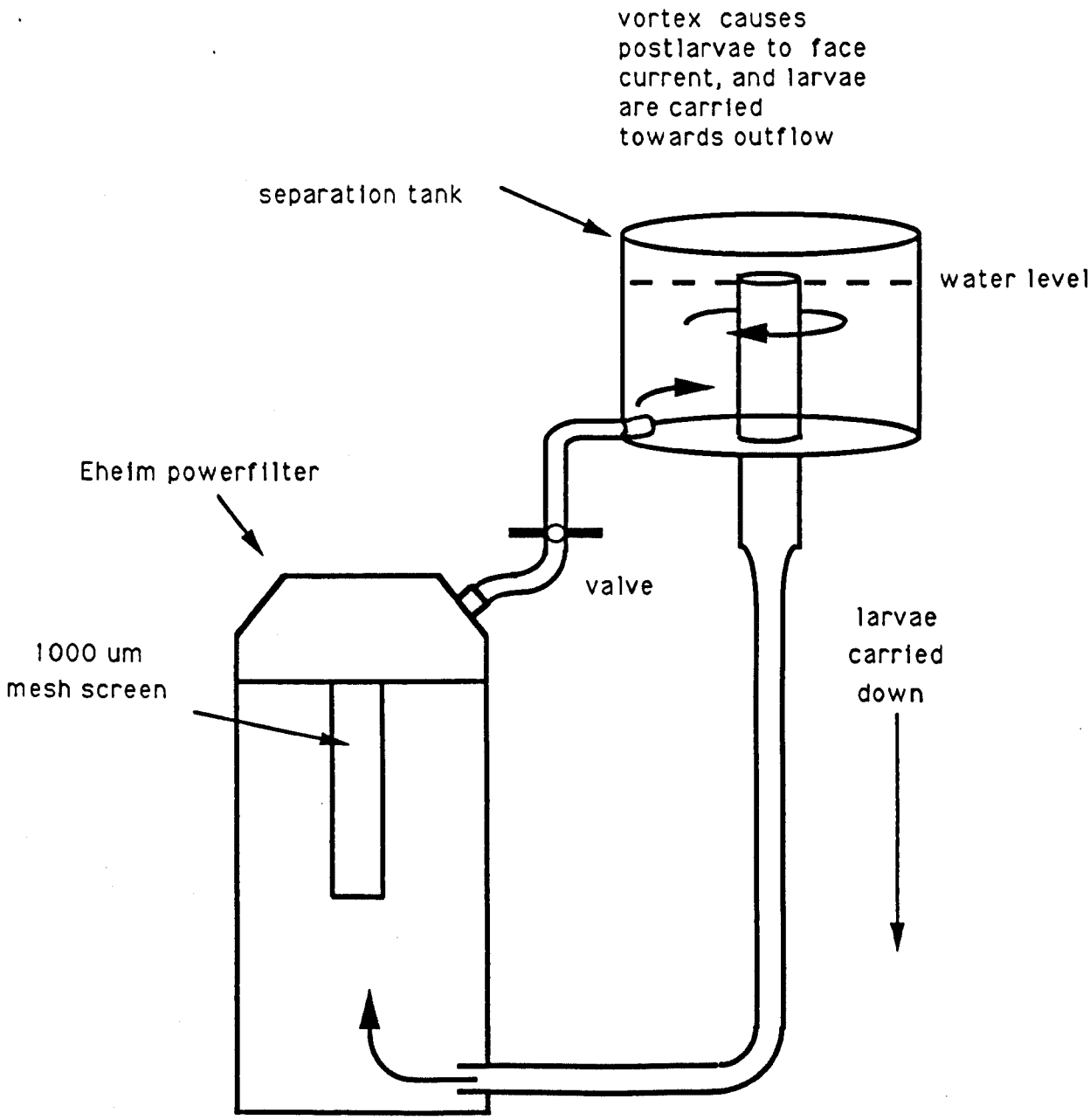


Fig.2 Larval/Postlarval Separator

3.2.9 Larval health

Larval condition was checked daily at the same time as the nutritional state was assessed. The principle indicators used were presence of fouling organisms, condition of setae, appearance of digestive gland and general pigmentation.

The fouling organisms found in the first rearing trials in static water were typical ectocommensals and bacteria (*Zoothamnion*, ciliated protozoa, and filamentous bacteria). With the improvement in water quality caused by the use of biological filtration these organisms were very rarely observed in later experiments. Occasionally in high density culture where the filter was overloaded (high nitrite and ammonia concentrations) the organisms might be observed. They were never present in high numbers and seemed to be lost as the larvae moulted. Larval mortality could never be attributed to disease in any but the preliminary culture trials. The success of the biological filters in controlling disease alleviated the need for sterilisation or disinfection procedures. Tanks and filters were cleaned between rearing cycles and the tanks were dried out. The use of antibiotics was never necessary.

Larval colouration was another indicator of the health and nutritional condition of the larvae. Early stage larvae are pale coloured and lack general pigmentation, although they possess several red chromatophores. As the larvae develop they begin to appear tan/amber coloured. Superimposed on the tan/amber colouration are red and blue chromatophores. It is interesting that when observed under reflected light the red chromatophores appear fluorescent green. Perhaps this explains the reported preference for green tanks (section 2.3.2). Stressed and diseased larvae appear a pale colour, or blue. This seems to be partly due to the lack of the tan/amber colouration emphasising the blue chromatophores, but the blue colouration itself seems increased. Newly moulted larvae also appear pale and torpid, often lying near the bottom of a beaker upon inspection and should not be mistaken for diseased or dying larvae.

3.3 Analysis of ions in adult and larval prawns.

3.3.1 Moult staging of prawns

Moult stages A, B, D_3', and D_3''' were determined in adult prawns according to the method given by Peebles (1979). His method of determination of other stages of the moult cycle relies on the evaluation of the degree of pigmentation of the prawns. This proved to be unsatisfactory for the prawns in these experiments due to their very pale pigmentation caused by the white tanks in which they were housed. Strong fluorescent lighting was originally present but this was subsequently changed in favour of green diffusers. Maintenance of the prawns under these conditions caused them to a darken, but not sufficiently to assess their degree of pigmentation with any degree of confidence.

For this work the moult stages sampled were defined as follows:

A - within 24 hours of moult.

B - over 48, but less than 72 hours after moult.

C - not less than seven or more than fourteen days after moulting.

D_3' - new setae visible within antennal scale.

D_3''' - exoskeleton pliable.

Prawns in intermoult (stage C) were determined by the length of time elapsed after moulting and the hardness of the exoskeleton. Intermoult prawns were exposed to experimental treatments not less than seven, or more than fourteen days after moulting. Time after moulting was known accurately since all the animals were checked daily and moulting was recorded. The sole use of carapace hardness as an indicator of moult stage is unsatisfactory as *Macrobrachium rosenbergii* remains quite hard until two days before moulting (moult stage D_3''- D_3'''), and it is therefore possible to sample erroneously premoult animals in the belief they are intermoult. It is possible that this is a frequent source of error in many pieces of work concerning crustaceans where carapace hardness is taken as the sole indicator of the intermoult stage.

3.3.2 Haemolymph sampling

Prawns were always starved for a period of two days prior to sampling. Haemolymph was withdrawn from the pericardial sinus using a 500µl 'Hamilton' gastight syringe. Volumes of haemolymph sampled varied depending upon the size of the prawn, but was generally between 200-450µl. The prawn was flexed to reveal the arthrodial membrane beneath the posterior cephalothoracic carapace. This was blotted dry prior to sampling. This method of sampling was selected since it allowed uncontaminated samples of haemolymph to be obtained rapidly with minimal disturbance to the prawns. Samples could be obtained in approximately 30 seconds from the removal of the prawn from the tank. This was particularly important when samples were to be used to determine the oxygen transporting properties of the haemolymph since it is because it is now well established that an increase in haemolymph l-lactate concentration can have a significant effect upon the oxygen affinity of haemocyanin (Morris 1990). Sampling from the pericardial sinus also prevented the possible contamination of samples with tissue or fluid from the stomach or hepatopancreas. The success of the method was seen in the almost 100% survival of the animals sampled. Those that died were usually in already stressful conditions such as high salinity exposure treatments, or stage D_3''' premoult. Initial attempts to obtain haemolymph samples from the ventral sinus were unsatisfactory. Samples were contaminated with digestive juice and tissue, the prawns also seemed traumatised by the technique. Later efforts were more successful, when haemolymph was drawn from the arthrodial membrane at the base of the third walking leg. Clean samples could be obtained quickly providing the syringe needle was not inserted too deeply.

3.3.3 Preparation and storage of haemolymph and serum

For the purpose of this work whole haemolymph and serum are defined as follows. Whole haemolymph is the haemolymph sampled from the prawn, and is not separated in any way. Serum was obtained from the haemolymph samples by centrifuging at 13,0000 rpm for 3 minutes. Any serum above the clot would be removed. The clot was disrupted with a pipette tip and the sample recentrifuged and the supernatant was removed. This process was repeated until a sufficiently large sample volume was obtained. Serum samples were stored in the 'Eppendorf' cuvettes in a domestic freezer (-20°C)

until required for analysis.

Samples for analysis using whole haemolymph were not centrifuged but weighed, dried at 60°C for 48 hours, and reweighed. Thus wet and dry weight values could be obtained for these analyses. The volumes of these samples were obtained either directly using the volume of the syringe, or indirectly by weight. Fieber & Lutz (1985) give the specific gravity of *Macrobrachium rosenbergii* haemolymph as 1.030. This is in good agreement with a value of 1.041 obtained using weighed samples from the 'Hamilton' syringe (250 µl), and the value of 1.030 for *Palaemon serratus* (Parry 1954).

3.3.4 Atomic absorbtion flame spectrophotometry (AAS)

Cation concentrations (Na, K, Ca, Mg, Sr, Cu) in adult haemolymph, acid digested larvae and seawater samples were analysed by Atomic Absorbtion flame Spectrophotometry (AAS). The AAS model used was a Phillips PU 9200.

The standards used for AAS were obtained as 1000 ppm concentration 'Spectrosol' grade (BDH). These were diluted to give the concentration range for the construction of calibration curves.

Since the technique of AAS relies on the linear relationship between absorbance and ionic concentration, samples were diluted such that the concentration of the ion to be analysed lay within the linear range for that ion. The dilution protocol for each ion was determined on preliminary samples of seawater, haemolymph and acid digested larvae. Dilutions of samples and standards were performed using a 'Hamilton microlab', 'Gilson' micropipettes, or by weighing. If weighing was used for any substance other than distilled water, account was taken of the specific gravity of the substance and corrections were applied.

In those experiments that utilised whole haemolymph samples it was necessary to digest the sample in concentrated nitric acid prior to analysis by AAS. This was because the rapid gelling of the whole haemolymph prevented dilution of the samples for atomic absorbtion spectrophotometry. Samples of larvae and postlarvae were also acid digested for ionic analysis using AAS.

Samples for acid digestion were placed in 'Eppendorf' tubes, acidified with 16N 'Aristar' (BDH) nitric acid, and sealed with PTFE tape. Samples were analysed within three days of digestion. It was found that all digested larval samples contained a small amount of insoluble material. This remained unidentified but it was assumed that this material was undigested chitin. No attempt was made to exclude it from samples, indeed, they were shaken to suspend the insoluble matter evenly.

The absorbance of each sample was read over a two second period after a period of stabilisation. The volume of sample used per reading was approximately 0.9 cm^3. This volume could be greater however, if the sample aspiration was set too high. When this occurred, the number of replicate readings per sample was severely reduced; in the case of strontium this might sometimes be to one. Whilst lack of replication of readings is undesirable, the larger sample volume being used by the AAS probably increased accuracy.

The analysis of concentrated samples often caused a rapid build up of contaminating matter on the burner head. This interfered with the flame and could give low readings if allowed to remain. This was easily visible as the development of irregularities in the eveness of the flame, and led to small gaps in the flame. Whenever these were seen to be forming, the AAS was stopped and the burner cleaned. The machine would then be recalibrated. This could occur as often as every four samples in the case of strontium, copper and calcium analyses. If the gaps in the flame were allowed to remain the calibration of the AAS would gradually drift, and low readings would result.

Lanthanum chloride (BDH, Spectrosol) was added to both samples and standards for calcium analysis (10% v:v). This was to suppress interference from other ions. Nitric acid was added to strontium standards since it affects the absorbance in acid digested samples (Brannon & Rao 1979).

3.3.5 Analysis of serum chloride

Serum chloride concentrations were measured using a Jenway PCLM3 chloride meter (sample volume 20 µl). This uses a potentiometric method for the estimation of chloride. Frequent cleaning of the electrodes and recalibration of the machine

ensured accuracy. Standards for calibration (100 mM) were supplied by the manufacturer.

3.3.6 Analysis of serum osmotic pressure

Serum osmotic pressure was measured using a Wescor 5500 vapour pressure osmometer. 10 µl samples were pipetted onto small discs of filter paper prior to loading into the sample chamber. Calibration of the machine was performed at frequent intervals using standards supplied by the manufacturer (100 mM). It was found that the thermocouple in the sample chamber needed frequent cleaning due to contamination by haemolymph.

3.3.7 Neutron activation analysis of bromine/bromide

As a result of its implication as an essential element in the successful rearing of larvae, the concentration of bromine/bromide in adult haemolymph, and larval and postlarval tissues was analysed using Neutron Activation Analysis (NAA). This technique involves the formation of short lived radioisotopes in a sample by exposure to a neutron flux in a nuclear reactor. Many short lived isotopes are formed but depending upon the intensity of the neutron flux to which the sample is exposed, and the time after exposure at which measurements are taken, the desired isotope can be analysed with a high degree of accuracy. In the present study, the isotope Br^{82} was used to determine the concentrations of bromine/bromide in haemolymph and samples of larvae and postlarvae. The half life of this isotope is 35.6 hours.

This method enabled the measurement of bromine/bromide in very small samples of haemolymph (250 µl) and larvae (0.020 g). Other methods of analysis for bromide were considered such as potentiometry and ion specific electrodes. They were rejected due to lack of accuracy, requirement of large sample volumes and susceptibility to interference from high chloride concentrations. NAA does have one major constraint as a method of analysis, which is that it does not give any information as to the chemical form of the element being studied. In this study it is unknown whether the form of the Br^{82} is present in the sample as organically bound bromine, as ionic bromide or both. The

irradiation time of samples and the decay period before analysis were established using preliminary samples of haemolymph and larvae.

3.3.7.1 Effect of salinity on haemolymph bromide concentration

Wet samples of whole haemolymph and of standards were placed in 'Eppendorf' tubes, weighed, dried at 60°C for 48 hours and reweighed. Activation analysis of the tubes showed them to contain less than 0.02 ppm bromine. After drying and weighing, the sample and standard tubes were wrapped in aluminium foil to reduce the effect of the heat from the reactor. Irradiation lasted for three hours with the reactor at full power, the neutron flux being approximately 3.8×10^{12} $n.cm^{-2}.s^{-1}$. A longer period of irradiation may have been desirable but there was a risk that the tubes might melt. After irradiation, samples were stored for 3.5 days, this allowed the majority of the radioactive sodium (Na^{24}) to decay (half-life 15 hours). This isotope was the principle interfering isotope in this analysis. The interference is caused by the very large amounts of Na^{24} produced in this irradiation procedure. As this decays it produces so much beta and gamma radiation that the sensitivity of the detector is reduced. The slower decay rate of Br^{82} meant that it was still sufficiently abundant after 3.5 days to be analysed, whereas the Na^{24} had decayed to a low background level. This time window after irradiation for Br^{82} analysis had the effect of limiting the number of samples it was possible to analyse. Only ten haemolymph samples could be processed per week.

Activated samples were analysed using gamma ray spectrometry. This was performed by a 25 cm^2, Germanium/Lithium (Ge/Li) detector linked to a computer driven 'EG&G ORTEC' multichannel analyser. This method of analysis utilises the fact that each decaying radioisotope produces a characteristic 'fingerprint' of gamma rays of different energies (KeV). The relative proportions of gamma rays at each energy is known for each isotope. By counting the number of decays per second at each energy the amount of each radioisotope in a sample can be determined. The inclusion of a standard containing a known concentration of bromide allows the calculation of the proportion of Br^{82} to total bromine to be established. This can then be used to calculate the concentration of bromine/bromide in the samples. The peak energies used in this analysis were: 554.3, 619.0, 698.3, 776.6, 1043.9, 1317.2 KeV.

3.3.7.2 Haemolymph bromide concentration during moult cycle, and larval bromide concentration during development.

Due to a six month failure at the SURRC research reactor at the National Engineering Laboratories, East Kilbride, bromine analysis was transferred to the Imperial College Reactor Centre, Ascot. This reactor differs from the SURRC reactor in that it operates at a lower power. This has the effect of producing a lower neutron flux of approximately 1.1×10^{12} $n.cm^{-2}.s^{-1}$. In order to activate the samples to approximately the same degree as at SURRC, exposure to the neutron flux was increased to 7.5 hours. This reactor operates at a cooler temperature and melting of the sample tubes was not a problem with this extended exposure. Samples were not wrapped in aluminium foil. Due to differences in the neutron flux geometry, standards were placed at the top and bottom of sets of samples and flux corrections were applied. However, these were found to be relatively small.

If the larval sample was too large, the total radiation from that sample (dead time) would still be high at the time of analysis, this was principally due to Na^{24}. This high radiation level caused an overloading of the counting system and thus resulted in reduced accuracy. This reduction in accuracy gave rise to unacceptable errors in the samples (± 30%) and they had to be rejected. If the radiation was principally ß particles, a 1.5 cm thick perspex block could be placed between the sample and the detector. However, if the high radiation level was due to Na^{24} then the gamma radiation could not be shielded out. The optimal weight of larval and postlarval samples was found to be approximately 0.02g. Haemolymph samples (250-350 µl) were always too small to cause this problem.

Sample counting times varied according to the actvity and dead time of the samples. The height above the detector at which samples were placed was dependent upon the dead time of the samples. All the samples irradiated at Imperial College reactor centre had very low dead times and were counted for 20 minutes at a height of 5 centimetres above the detector. The samples that were analysed at SURRC were counted at heights between 7-10 centimetres for a period of 30 minutes. The higher dead times of these samples necessitated a longer counting period to ensure accuracy. Samples with dead times in excess of 10% were not used. This was only found in preliminary analyses of larval material.

Standards for bromide analysis were prepared using 'Analar' potassium bromide. This was dissolved in distilled water in a volumetric flask to yield a 40 ppm solution. This standard solution was used in all of the analyses. An 'Eppendorf' tube was weighed then filled with standard and reweighed. The volume of standard was calculated by weight. For both haemolymph and larval samples a standard volume of ≈250 µl was used. For larval samples, the standard was pipetted onto alpha-cellulose to present the standard in the same spatial form as the larval samples (J. Whitley 1990 pers. comm.). Thus the volume of alpha-cellulose corresponded approximately to the same volume as the larval samples. Analysis of the alpha-cellulose showed it to have a bromide content of approximately 1.25 ppm bromine. This was usually less than 1%, and not more than 2% of the bromine detected in the larval samples.

CHAPTER 4 - Seawater as a culture medium.

This chapter considers the composition of natural and artificial seawaters in relation to their ability to support the growth of larval *Macrobrachium rosenbergii.* The results of this work would certainly apply to other brackishwater crustaceans, and possibly other brackish and marine species.

4.1 Seawater composition

> *"The principle of constant ratios between the salts in ocean water unaffected by land drainage was laid down by Forchhammer in 1864, and firmly established by Dittmar (1884)."*
>
> (Lyman & Fleming 1940)

In this statement Lyman & Fleming (1940) are referring to what are known as the "major ionic constituents" of seawater. The "major ionic constituents" are those elements which make a significant contribution to the salinity of seawater. All are present in seawater at concentrations greater than 1 $mg.kg^{-1}$ (ppm). The concept of the constancy of composition of seawater regarding the major ions is not strictly correct, for all workers who have studied seawater composition have reported slight variations in seawater samples from different areas and different depths (Culkin 1965). However, the variations between the major ions are sufficiently small to enable the ratios between the ions to be determined and used with a reasonable degree of accuracy. Analyses of the concentrations of the major ions from around the world are given in Culkin (1965).

Table VI contains six different compositions for oceanic seawater. All the values in the following table have been converted to a seawater of 35 $^{o}/_{oo}$ salinity and are expressed in $mmol.kg^{-1}$ of seawater.

TABLE (VI) Concentrations of selected ions and elements in seawater at 35 °/ooS - values in (mmol.kg^{-1}).

ION	YEAR OF PUBLICATION					
	1934[a]	1940[b]	1954[c]	1965[d]	1965[e]	1973[f]
Na^+	462.860	468.126	468.139	468.034	465.640	468.016
K^+	9.790	10.167	9.911	9.900	9.911	10.207
Ca^{2+}	10.390	10.177	10.180	10.304	10.175	10.277
Mg^{2+}	53.310	53.356	53.367	53.251	56.640	53.343
Sr^{2+}	0.15300	0.15480	0.15520	0.09130	0.09310	0.08850
Cl^-	545.440	545.802	545.802	545.878	545.660	545.870
SO_4^{2-}	85.500	28.111	28.132	28.250	28.140	28.240
HCO_3^-	-	2.334	2.333	2.328	2.379	2.315
Br^-	0.82600	0.82430	0.82420	0.83850	0.82940	0.84380
F^-	0.04380	0.06980	-	0.05260	0.06980	0.06830
B	0.03772	-	0.07504	0.37002	0.43384	0.02550
Si	0.0408				0.1089	
Li^+	0.01529				0.0250	
Fe	0.00367				0.000183	
Mn	0.003059				0.0000371	
P	0.00204				0.00230	
Cu	0.002				0.000862	
I	0.000357				0.000482	
Zn	0.0000306				0.00190	
Cr					0.00000098	
V					0.00004	
Co					0.00000173	
Rb					0.00143	
Mo					0.000106	

a) Thompson & Robinson (1934)
b) Lyman & Fleming (1940)
c) Barnes (1954)
d) Culkin (1965)
e) Goldberg(1965)
f) Morcos (1973)

The minor elements of seawater are far more variable in their concentrations, This is due mainly to their low abundance coupled with their involvement in many biological processes and their high reactivity. The source rocks or subsea gaseous emanations that replenish depleted waters with these elements do not contain them in great concentrations and hence the rate of replenishment is slow. If a minor element is removed rapidly from the environment then it will only ever be present at low concentration. An exception to this case is aluminium, one of the most abundant elements on earth, yet present at very low concentrations in seawater. This element is so reactive that it is rapidly removed from solution thus maintaining it at a low concentration (Goldberg 1965)

The concentrations of minor ions are enhanced in areas where freshwater runoff carries leached minerals into the sea. Areas of coastal upwelling cause the transport of minerals and nutrients from the unproductive deeper waters to the surface. At the surface they are available to bacteria and phytoplankton which require these elements for growth. The growth of these organisms is often limited by the low concentrations of the trace elements. The transport of nutrients and sediments into estuaries and coastal waters by rivers, and deepwater upwelling gives high local productivity of both phytoplankton and subsequently zooplankton. It is no surprise that many species have larval and juvenile phases that exploit these areas of high productivity and have developed behavioural mechanisms by which their young are delivered into regions of high productivity, or are hatched at periods of phytoplankton or zooplankton abundance.

4.2 Trace element requirements

According to Underwood (1977) the trace elements found in nature can be divided into three groups: essential, possibly essential and non-essential. The non-essential trace elements are those which are found in animals in variable concentrations, and are thought to be acquired by the body as environmental contaminants. Their concentration therefore reflects the degree of exposure of the organism to the contaminant. Underwood (1977) cites the criteria of Cotzias (1967) for the consideration of an element as essential:

1) It is present in all healthy tissues of all living things.

2) Its concentration from one animal to the next is fairly constant.

3) Its withdrawal from the body induces reproducibly the same physiological and structural abnormalities regardless of the species studied.

4) Its addition either reverses or prevents these abnormalities.

5) The abnormalities induced by deficiency are always accompanied by pertinent, specific biochemical changes.

6) These biochemical changes can be prevented or cured when the deficiency is prevented or cured.

Elements which fulfill these criteria in animals are carbon, hydrogen, oxygen, nitrogen, sulphur, calcium, phosphorus, potassium, sodium, chlorine and magnesium. These elements are present in high concentrations in the body and can be called the major elements. The essential 'trace elements' are iron, zinc, copper, manganese, nickel, cobalt, molybdenum, selenium, chromium, iodine, fluorine, tin, silicon, vanadium, and arsenic (Underwood 1977).

The 'possibly essential' trace elements are those which seem to be essential by virtue of their ubiquitous presence in living organisms, but for which insufficient information is available to adequately classify them. Reinhold (1975) lists these as, arsenic, barium, boron, bromine and strontium.

Underwood (1977) gives the classification of Venchicov (1974) of the effects of trace elements on an organism. These effects can be defined into three zones of action depending upon the dose an organism receives. In order of increasing dose these are:

1) Biological action zone: as the dose increases there is an increasing effect leading to a plateau which represents the optimal level for normal function of an organism.

2) Inactive zone: this dose range does not exert any additional effect on the organism. The extent of this dose range depends upon

the organisms' homeostatic ability to maintain the desired concentration of the element. The level of the trace element within the organism should not vary significantly.

3) Pharmacotoxicological zone: this dose range begins with the irritation and stimulation of some function, the pharmacological effect; and at higher doses the element becomes toxic to the organism, the toxicological effect.

The dose levels for these three zones will vary both between organisms and between trace elements. Reinhold (1975) classifies the essential trace elements as being those which are essential to higher animals. This would seem to be appropriate as the majority of trace element research has been concerned with humans, mammals and birds. The reason for this is that the predominant interest in trace element research has been in the fields of human nutrition, medicine and agriculture.

The bulk of the research that has been conducted as to the essentiality of trace elements in marine systems has been concentrated on the requirements of marine algae. This is due to the difficulty of maintaining monocultures of marine phytoplankton in the laboratory, necessitating reproducible methods. Due to the variability of coastal seawater, it is desirable for much of this work to be performed with defined artificial media. The work involving trace elements and marine animals has largely involved analysis of specimens for the presence of those trace elements. This is a valid method for determining the availability of the element to the animal, although the presence of an element does not immediately suggest that it has a role in that animal's physiology. As has been mentioned previously, non-essential elements can be stored or excreted and their presence and concentration often reflects the degree of environmental exposure of the animal.

A second problem that arises in the study of the essentiality of elements to animals in marine systems is environmental contamination. The method by which the essentiality of trace elements is evaluated in terrestrial animals is by the use of plastic isolators. These isolators attempt to exclude all environmental contamination by the trace element under study. This is achieved by the use of filtration of air to exclude bacteria and dust, the feeding of purified diets and solute-free water. The diets consist of elements not

under study and crystalline amino-acids. When studying aquatic and marine animals, the organism is immersed within the medium and thus continually in contact with it. Many of the elements that are required by the organism are absorbed directly from the water, any shortfalls being obtained from the diet. In the case of seawater, all the elements must be supplied, except the element under study, in order that the effect of deprivation of that element can be observed. All the salts to be used for this type of experiment would have to be of a very high purity. Even if this were possible, the feeding of an inert purified diet is not, since the absolute dietary requirements for marine organisms are unknown. It therefore becomes necessary to compromise as to how the essentiality of an element is to be determined. The fact that seawater supplies trace elements from the medium means that these can be largely ignored providing there is an abundant supply of seawater to the organism. Thus the avenue of nutritional research in fish, crustaceans and molluscs has tended to provide generalised mineral mixes in artificial diets. It is not unknown for fish and prawn diets to be supplemented with chicken mineral and vitamin mixes. The requirement for essential elements in the diet becomes more important in freshwater, where the concentrations of all ions are low. The ability to absorb these elements from the environment must become difficult if not imposssible due to their very low environmental concentration. Good examples of such elements are fluorine, bromine and iodine. All of these elements are abundant in seawater relative to freshwater, yet all are found to some degree in freshwater organisms. Thus, freshwater organisms need to concentrate these elements to a much higher degree than the marine/brackishwater species.

If a significant biological effect cannot be obtained by limiting an element in seawater, then some idea of its effect might be obtained by providing it in excess. This would put the animal under study into the zone of pharmacological action for that element. This is not difficult to do as the normal environmental ranges of most of the trace elements are well documented. The method by which the biological zone of action of a trace element is often determined is by the use of growth trials. As the concentration of an essential element increases the growth of the animal is often enhanced. When studying pharmacotoxicological effects this is not possible. Growth may be suppressed due to a toxicological effect and so any pharmacological effect may be less easy to observe.

Another approach in determining the biological zone of action is by the analysis of body tissues and fluids. A problem arises, however, in that tissue concentrations in terrestrial animals largely reflect diet, whereas in aquatic and marine

animals the environment has a significant effect as well as dietary sources. What can be determined is how a species regulates an element at different doses. For a marine organism this can be done in two ways:

1) Exposure to different concentrations of an element at a fixed salinity.

2) Exposure to different concentrations by exposure to differing salinities, such that the element under study is in fixed ratio to the other seawater constituents.

Which method to use is determined to some extent by the animals' range of salinity tolerance and the element under study. In the case of a stenohaline organism, or one of the minor trace elements of seawater (see section 4.1), method 1 would be employed. If the organism has a wide range of salinity tolerance, or if bromine, fluorine, strontium, boron, (possibly lithium) was being studied, either method could be used; each has advantages. Method 1 studies the animals' ability to regulate differing environmental concentrations and would be preferable when studying elements present at low concentration in seawater. This enables a wide range of concentrations to be tested that would not be possible using varying salinities of seawater. Method 2 allows the relationship between the essential element under study and other elements in the blood to be observed, as the salinity changes. This is especially the case with *Macrobrachium rosenbergii* since this prawn can tolerate a salinity range of 0-28 $^{o}/_{oo}$. The ability to tolerate freshwater and low salinity is especially important as it is here where the normally steady ratios between the major constituents of seawater (Na, Ca, K, Mg, Sr, Cl, SO_4, HCO_3, B, Br) begin to break down. It should be noted that bromine, strontium, and boron, are all thought to be essential trace elements to terrestrial animals, but are usually present in high concentrations in the environment so that deficiency-related problems have not been identified. Similarly, the concentrations of these elements in seawater (35 $^{o}/_{oo}$S) are all in excess of 1mg.kg^{-1} (ppm). However, their concentrations decrease with the increasing dilution of seawater and it is possible that a threshold may exist whereby the animal can no longer satisfactorily obtain the required element from the environment.

4.3 Dilution of seawater

If full strength seawater (35 °/oo) is diluted with deionised water then the ionic ratios found in seawater are preserved. This is especially the case with the major constituents. The chemical speciation of some of the minor constituents may change as equilibria are shifted in the diluting conditions. This will not be considered since the form of the major elements in seawater is as the free ion (Goldberg 1965). Dilution of seawater occurs principally in areas where there is a high degree of freshwater input. These areas are predominantly estuaries, deltas and coastlines, although heavy rainfall can affect surface water temporarily. Only water of terrestrial origin will be considered as this contains the most dissolved elements. The dilution of seawater with river water does not give the constant ionic ratios seen with deionised water. This is due to the concentrations of calcium and magnesium in freshwater runoff being greater than those of sodium and chloride. Whilst freshwater contains only very low ionic concentrations they are sufficiently great to cause a significant change in the ionic ratios in brackishwater at salinities below 2 °/oo (Deaton & Greenberg 1986). Freshwater usually contains more calcium than magnesium, whereas the reverse is true for seawater, which has a Ca:Mg ratio of 1: 5.19.

In an estuarine environment, the dilution of seawater may cause the concentration of an essential trace element to decrease below the threshold level at which it is required by an organism. It may be that the organism is able to obtain sufficient from its food, in which case it would be relatively unaffected. However, if that organism required the essential element at an environmental concentration for the correct functioning of a physiological process, or was unable to derive it from its food then there may be a salinity below which it was unable to survive, even though it was capable of surviving the osmotic stress.

Deaton & Greenberg (1986) consider the reasons for the low numbers of species found in brackishwater at salinities between 5-8°/oo. They propose that the low species diversity is due to the limited numbers of organisms that have evolved the physiological mechanisms to withstand a variable habitat, and that those species which have such mechanisms have low rates of speciation. They do not consider the possibility of trace element dilution, but do mention the existence of a physiological barrier between

brackishwater species and freshwater species at a salinity between 0.5 to 1 $^{o}/_{oo}$ (Deaton 1981). One reason for this might be the change in Ca:Mg ratio affecting Na/K ATPase function in the animals. Winkler (1986) found that in the presence of 1.5 mmol.dm^{-3} calcium, a magnesium concentration of between 25-50 mmol.dm^{-3} was required to prevent inhibition of Na/K ATPase in *in vitro* preparations of gills from the shore crab *Carcinus maenas.*

4.4 Artificial seawater

One of the best known artificial seawaters ever devised is that formulated by Lyman & Fleming (1940). In this paper the authors presented a formula for the composition of seawater that could be made from soluble salts. When made up with distilled water it would give ionic concentrations for sodium, potassium, calcium, magnesium, strontium, chloride, sulphate, bicarbonate, bromide, fluoride, and boric acid, similar to those those found in seawater. The composition of this seawater approximates to the oceanic seawater found anywhere in the world and as such could be used in experiments involving temperate or tropical marine species.

The quotation at the beginning of section 4.1 has one proviso, that the oceanic seawater must be unaffected by land drainage. This means that any work involving coastal or estuarine species must take into account the fact that freshwater runoff from the land can alter the composition of seawater. This is especially the case with estuarine environments where appreciable dilution of seawater by freshwater occurs.

There have been many different formulations for artificial seawaters as various workers have tailored them to suit their individual research or culture purposes. The majority of those recorded can be found in the book "Artificial Seawaters" by Bidwell & Spotte (1985). One of the factors that has caused a progressive change in the formulations has been improved methods of analysis for the elements, strontium, bromide and fluoride. Apart from small changes in the concentration these elements, the major ion formulation of artificial seawater has varied little since the time of Lyman & Fleming (1940).

The major difference found between the various artificial seawaters and culture media is the concentration of trace elements. Marine algal, bacterial and yeast culture media have exotic compositions that include levels of trace elements far in excess of those found naturally , even in coastal and estuarine waters. It is the low abundance of some of the trace elements which is thought to limit algal productivity in the oceans (e.g. cobalt and vitamin B_{12} formation, Tait 1968). By including these elements in excess together with a suitable chelator (e.g. Na_2EDTA, sodium citrate), algal production is not limited with respect to these elements and thus growth is enhanced.

Since the concentration of trace elements varies so widely, it is difficult to determine an appropriate concentration for each element in an artificial seawater unless the requirements for a particular species are known. Many of the general formulae for complete seawaters, especially commercial aquarium formulae, appear to contain concentrations for trace elements which have been decided upon arbitrarily. Provided the trace elements are not present at toxic levels and a chelating agent is present, the inclusion level seems to be irrelevent. This is exemplified by the change in formulation of the commercial aquarium seawater "Instant Ocean". Early formulations of this seawater claimed an exhaustive set of trace element inclusions, although the formulations were not made available. With time, the formulation gradually changed until in 1980 a full formula was given. This contained only the major salts found in seawater. The rationale is that since technical grade salts were used in its manufacture the level of trace element impurities would be sufficient to provide the levels required by marine organisms (Bidwell & Spotte 1985). It is interesting that bromide, fluoride and strontium are not included, even though they are classified as major ionic constituents of seawater. The assumption to be drawn from this is that they are considered unimportant or alternatively, are adequately provided as impurities. In the case of bromide and strontium this is somewhat questionable, for their concentrations in seawater are high enough to allow them a fixed ratio with other major ions, and it would be desirable to ensure that they were present in accordance with that ratio. It might be that bromide is considered inessential and as such not worthy of consideration. This is not an unreasonable view as little evidence exists to demonstrate its essentiality. There is little published evidence of the essentiality of strontium to animal life with the exception of a few species of diatoms which have a test constructed entirely of strontium salts, although the partial replacement of calcium by strontium is well documented (Vinogradov 1953; Dodd 1967; Gibbs &

Bryan 1972). Bidwell *et al.* (1986,1990) report that strontium appears to be essential for the normal embryonic development of several species of mollusc.

Various attempts have been made to rear the larvae of *Macrobrachium rosenbergii* in artificial seawater media (Minamizawa & Morizane 1970; Sandifer & Smith 1974 in Tansakul; Tunsutapanich 1981; Tansakul 1983; Yambot & Vera Cruz 1986; Prakash 1988; Prakash *et al.* 1987; Lovatt & Felder 1988) and all have had some degree of success, depending upon how complex the composition of the media. The most interesting formulation was that of Tunsutapanich (1981) in which he successfuly reared postlarvae in a combination of rock salt and salt pan residue. This residue contains a strong solution of many of the less abundant elements found in seawater. Unfortunately, the composition of this solution is unknown, since the salts that have crystallised out are lost when the residues are reconstituted. A salinity of 350 $^{o}/oo$ is given for the residue used in this work, and the approximate constitution of this seawater can thus be calculated from the analyses performed by McCaffrey *et al.* (1987). These authors studied the evaporation of seawater and analysed the composition of the brines that were left as the seawater evaporated (Table VII). It is not possible to calculate the ionic content of salt pan brines using just the salinity, since the degree of evaporation determines which salts are crystallised out of solution and which of those are left. McCaffrey *et al.* (1987) state that calcium carbonate begins to precipitate at a brine concentration of 1.8 times that of seawater, gypsum ($CaSO_4.2H_2O$) at 3.8 times and halite (NaCl) at 10.6. The brine that has an ionic content of approximately 350 $^{o}/oo$ (350,000 mg/litre) is concentrated 25.4 times normal seawater and has the following composition of major ions:

Table (VII) Concentrations of ions in a salt pan brine of salinity approximately 351$^{o}/oo$ - calculated from McCaffrey *et al.* (1987).

	Cl^-	Br^-	SO_4^{2-}	Mg^{2+}	Ca^{2+}	K^+	Na^+
$mg.dm^{-3}$	192000	1390	40900	30300	-	8800	78000
$mmol.dm^{-3}$	6228	19.97	490	1434	-	259	3902

It is readily apparent that the brine at 351 °/ooS does not contain any calcium, and by implication, very little bicarbonate or carbonate, since these would have precipitated with the calcium. A rearing medium made by rediluting this brine and adding rocksalt to replace the lost halite, would still have a composition similar to that of seawater with the exception of the concentration of calcium. Although calcium would be present in the diluting freshwater, the pH of the resulting artificial seawater would probably be low, and the water would not have a good buffering capacity due to the low carbonate/bicarbonate content. This could easily be rectified by the addition of sodium bicarbonate ($NaHCO_3$). The overall survival in this medium was 42%, giving about 10 postlarvae per litre (initial stocking density 25/litre) (Tunsutapanich 1981).

Cawthorne *et al.* (1983) attempted to acclimate juvenile *Penaeus monodon* to low salinity (≈1.7°/oo) in a variety of salt solutions. Their conclusions were that the solutions containing only one salt could not support the animals at all, and that more complex artificial seawater solutions containing mixtures of salts were still slightly inferior to natural seawater. Other workers who reared larvae in commercial and formulated seawaters have also had success. Lovatt & Felder (1988) commented upon the fact that starved *M. rosenbergii* larvae reached a slightly more advanced stage in artificial seawater than natural seawater before dying (mean stage 2.98 and 2.33 respectively). Nevertheless, the use of formulated artificial seawaters is generally ignored as a solution to rearing inland in a low technology environment. This is due to the high cost of chemicals and their generally limited availability. It would not be impossible for a fisheries station to obtain the necessary materials for a hatchery using artificial salts and supply postlarvae to local farmers. It is the expense of the artificial seawater that seems to prevent this occurring. The high cost of artificial seawater media can be offset by the use of recirculation systems, although these do need a high degree of supervision. What could be envisaged is a government funded hatchery providing postlarvae using artificial seawater media and recirculation in order to stimulate local growout operations.

The demonstration that *Macrobrachium rosenbergii* postlarvae can be successfuly reared in artificial seawater allows the possibility of omitting selected elements from the artificial seawater to establish whether they are essential to the developing larvae. If the elements that are not required can be omitted, it should be possible to produce a basic rearing medium for the culture of *Macrobrachium rosenbergii*

postlarvae. This basic medium would have the advantage of being cheaper than the commercial formulations, yet give comparable survival and growth to that achieved in natural seawater. The formulation of the artificial seawater would also have to support the larvae with minimal water exchange, thus the trace elements it contains cannot be depleted before the completion of larval metamorphosis.

The aim of this section was to design and test a rearing medium suitable for the culture of *Macrobrachium rosenbergii* past metamorphosis. Once this had been achieved the intention was to minimise the number of constituents in this medium. In order to minimise the volume of artificial seawater used, recirculation and biological filtration of the rearing medium were employed.

4.5 Materials and methods

Artificial seawater formulation

In order to test whether any trace elements might be essential to the larvae of *Macrobrachium rosenbergii* when reared in artificial seawater, an artificial seawater was designed using a computer spreadsheet. This performed rapid calculations of total individual ionic concentrations when the concentration of different salts was varied. The spreadsheet listed 21 compounds, and depending upon the inclusion level chosen would update the concentrations of the 22 ions under study. The concentration of the various elements found in seawater were taken from a variety of sources. In the case of the trace elements a range of concentrations is usually given, and arbitrary values were taken within that range, corresponding to Pacific ocean water if possible.

8 compounds were used to formulate an artificial seawater stock. This contained the major ions in the proportions found in seawater (Na, K, Ca, Mg, Cl, SO_4, HCO_3, H_3BO_3) and a chelating agent (Na_2EDTA). The starting point for the relative proportions of the 8 compounds used in the stock solution was the artificial seawater formulated by Kester *et al.* (1967). The final composition of this stock solution (Table VIII) still largely resembles their artificial seawater. Trace element solutions were prepared individually and added to test solutions using a 'Gilson' micropipette. This enabled

individual trace elements to be omitted from different test solutions. Na_2EDTA was added routinely to chelate trace metals. Licop (1983) found that routine addition of Na_2EDTA to the rearing water of larval *Penaeus monodon* improved survival. Her conclusion was that chelation of potentially toxic heavy metals in the rearing water gave the improvement in survival. The calculated composition of the formulated artificial seawater is given in table IX.

Composition and preparation of artificial seawater

The chemicals used in the preparation of the artificial seawater stock were general purpose reagents and technical grade reagents (BDH Chemical Co. Ltd.). Analytical grade reagents were not used since their cost would have proved prohibitive. The use of these lower quality chemicals was reflected in the results obtained from the various seawater trials. The decision to use general purpose reagents was made in the knowledge that if the seawater formulation were to be used in the field, the purity of the salts would be inferior. Thus any trace elements found in the general purpose reagent would certainly be found in technical and crude salt preparations. The use of general purpose grade sodium chloride in early trials gave satisfactory results. Later use of technical grade sodium chloride gave less definite results (see section 5.4).

Table (VIII) Composition of Artificial seawater stock (ASW) and trace element additions.

	(For salinity = 35 $^0/_{00}$)	
	compound	**inclusion ($g.dm^{-3}$)**
Stock solution	$NaCl$	23.8880
	$MgCl_2$	5.0788
	$CaCl_2$	1.1405
	KCl	0.6981
	$NaHCO_3$	0.1944
	H_3BO_3	0.0255
	Na_2SO_4	4.0107
	Na_2EDTA	0.00005

	compound	inclusion ($g.dm^{-3}$)
Trace elements	$SrCl_2.6H_2O$	0.023730
	KBr	0.100400
	NaF	0.002874
	LiCl	0.001014
	KI	0.0000785
	$MnSO_4.4H_2O$	0.0000079
	$NaH_2PO4.2H_2O$	0.0003440
	$Na_2MoO_4.2H_2O$	0.0000227
	$ZnSO_4.7H_2O$	0.0000429
	$CoSO_4.7H_2O$	0.0000023
	$CuCl_2.2H_2O$	0.0000078
	$VOSO_4.H_2O$	0.0000069
	RbCl	0.0001657

The artificial seawater stock was prepared by addition of the constituents to a 100 litre polyethylene tank containing deionised water (approximately 65 litres). Once dissolved, the salinity was determined by the use of a refractometer and adjusted to give the desired salinity. The seawater stock was not prepared at 35 °/ooS, but at approximately the desired salinity of 12 °/oo. The trace element solutions, with the exception of fluoride, bromide and strontium, were all made up such that the addition of 10 µl per litre of seawater gave the trace element in the desired concentration. The fluoride, bromide and strontium solutions were made up such that the addition of 250 µl per litre gave the desired concentration. This was necessary due to their much greater concentration in seawater affecting their solubility.

Once prepared the artificial seawater stock was aerated and heated to 28°C. Evaporation during storage caused an increase in salinity, and this was adjusted by the addition of deionised water.

Table (IX) Ionic concentrations in natural and artificial seawater at a salinity of 35°/oo. *

Ion	artificial mmol.kg^{-1}	natural mmol.kg^{-1}
Na^+	468.3010	468.0163
K^+	10.2079	10.2076
Mg^{2+}	53.3426	53.3429
Ca^{2+}	10.2761	10.2770
Sr^{2+}	0.0890040	0.0890208
Li^+	0.0239185	0.0239101
Mn^{2+}	0.0000354	0.0000355
Zn^{2+}	0.0001492	0.0001493
Co^{2+}	0.0000083	0.0000083
Cu^{2+}	0.0000458	0.0000460
Mo^{4+}	0.0001014	0.0001017
Rb^+	0.0013703	0.0013701
V^{4+}	0.0000381	0.0000383
Cl^-	545.5445	545.8748
SO_4^{2-}	28.2383	28.2372
HCO_3^-	2.3141	2.3141
H_3BO_3	0.4126	0.4124
Br^-	0.8436812	0.8435122
F^-	0.0684478	0.0684268
I^-	0.0004729	0.0004728
P	0.0022050	0.0022051

* values for artificial seawater obtained by calculation.

4.6 Experiments

The first trials with the artificial seawater attempted to establish whether it would support growth and development of the larvae through to metamorphosis. The seawater used was the complete medium. If this was found to be satisfactory then further trials would be performed excluding a different trace element in each treatment. These trials were difficult to perform for a number of reasons, and hence were used as pilot studies.

4.6.1 Experiment 1 - Formulated artificial seawater as a rearing medium.

The first aim of this experiment was to test whether or not the complete artificial seawater formulated (section 4.5) would support larval growth beyond metamorphosis. The second aim was to see whether a recirculation system would support larval growth yet keep water changes to a minimum over the course of a larval rearing cycle. These are two basic requirements of an artificial seawater hatchery.

This trial was the first performed using the complete artificial seawater solution and the recirculating biological filter. One rearing tank contained real seawater at 12 $^{o}/_{oo}$S, the other contained the complete artificial seawater. The method of rearing is described in section 3.2. The artemia used were enriched with SELCO. Stage I larvae were stocked in each tank at a density of approximately 100.dm^{-3}. The temperature varied between 28-30 ^{o}C, but was generally at 30 ^{o}C.

Uneaten artemia were removed daily from the rearing tanks and fresh artemia were added afterwards. To avoid possible problems with nitrite accumulation, each treatment had a second separate recirculating tank containing the same volume of the same type of rearing water. The water in the rearing tank was exchanged daily with that of the recirculating tank. By this method nitrite did not reach a sufficiently high concentration to present a problem.

Results and discussion

This initial experiment was not totally successful since the aeration failed in the artificial seawater tank causing the death of all the larvae on day 15. The larvae from the tank containing natural seawater were then split between the two tanks. The results in table X show that larval development in the two treatments was comparable.

The larvae in the artificial treatment passed through metamorphosis more quickly than those in natural seawater, whether this was due to the seawater or a combination of factors is uncertain. In both treatments the health and condition of the larvae was excellent.

Despite the problems encountered these results showed that the artificial seawater could support larval growth past metamorphosis, and that recirculation could remove the need for water exchange over the whole rearing cycle.

Table(X) Rate of development of larvae in natural and artificial seawater.

	Larval stage	
	Artificial	natural
DAY	seawater	seawater
1	1	1
2	2	2
3	2	2
4	3	2/3
5	4/5	4/5
6	5	5
7	5	5
8	5	5
9	6	6
10	6	6
11	7/8	7/8
12	8	7/8
13	8	7/8
14	8	8
15	air off	-
17	8	8
19	9	9
20	9	9
21	10/11/pl	10/11/pl
28	all pl	pl,many larvae
31	-	pl,still many larvae
34	-	"

4.6.2 Experiment 2 - The effect of trace element omission from artificial seawater on larval survival.I.

This preliminary experiment was designed to show whether the exclusion of an element from the complete artificial seawater would lead to the death of the exposed larvae. If this were to happen then this element could be considered vital for larval growth/survival. Ten larvae at stage 5 were placed in 500 cm^3 glass beakers. Each beaker contained artificial seawater stock together with all but one of the trace elements, a different trace element being excluded in each one. Additional beakers contained artificial seawater stock, with all the trace elements or natural seawater controls. A low stocking density was used in this experiment to discourage cannibalism. The beakers were aerated and placed in a water bath at 28°C. This trial lasted for five days and the larvae were unfed.

Results and discussion

Table (XI) The effect of trace element omission on larval survival, when maintained in artificial seawater.(Larvae unfed).

	Missing element	N° stocked	N° after 5 days	
1	fluoride	10	7	
2	bromide	10	0	
3	strontium	10	6	
4	lithium	10	9	
5	manganese	10	7	
6	copper	10	7	
7	molybdenum	10	4	
8	iodide	10	6	
9	cobalt	10	7	
10	zinc	10	7	
11	rubidium	10	6	
12	seawater[1]	10	5	**control**
13	seawater[1]	10	5	**control**
14	ASW stock[2]	10	0	
15	ASW stock[2]	10	0	
16	ASW stock[2]	10	0	
17	ASW+traces[3]	10	3	**control**
18	ASW+traces[3]	10	6	**control**

[1] seawater control at 12 °/ooS, [2] artificial seawater stock, [3] artificial seawater stock and all trace elements (control).

The results from this trial were quite inconclusive (Table XI) due to the disappointing larval survival in the four control treatments. It was felt that the design of the experiment was inadequate due to the limited time the larvae were exposed to the test seawaters. Since the larvae were unfed there was a natural constraint on the duration of the experiment. This was because the larvae would either starve or cannibalise each other. Cannibalism might have been the cause of the low survival in the control treatments. This loss of larvae is not satisfactory in this experiment which relies upon survival to denote whether an element is essential or not. In those treatments where there was not total mortality it was concluded that either the larvae had no requirements for the trace elements, or that they were spending insufficient time in the exposure medium to develop a deficiency. Another possibility was that the artificial seawater stock contained sufficient concentrations of some trace elements to support the larvae.

Although there was mortality in all treatments, dead larvae were not always found, and it was assumed that those lost had been cannibalised. In the treatments where no larvae survived this was not the case, and dead larvae were found on the bottom of the beaker (ASW stock, ASW + all trace elements except bromide). It was thus tentatively concluded that artificial seawater stock alone could not support larvae, and that artificial seawater stock with all the trace elements except bromide also could not support larvae.

Due to the disappointing survival in the control treatments this experiment was not repeated.

4.6.3 Experiment 3 - The effect of trace element omission from artificial seawater on larval survival. II.

The aim of this experiment was the same as experiment 2 (4.6.3). As a response to the problems encountered in experiment 2, this experiment utilised recirculating aquaria to allow the feeding of the larvae, without changing the test media. It was hoped that this would reduce the cannibalism that seemed to occur in the last experiment. The use of recirculation was intended to allow the provision of sufficient artemia to reduce this, and maintain good water quality.

All the tanks, except the seawater control contained artificial seawater stock (ASW), lithium, phosphate and copper. Larvae were stocked at stage 2/3 (predominantly stage 2) at a density of 100 per tank.

Results and discussion.

Table (XII) The effect of trace element omission on survival of larvae reared in artificial seawater with recirculation. (Larvae fed).

	SURVIVAL TIME (Hours)		
	48	72	96
Controls			
1 seawater[1]	78	55	52
2 ASW+traces[2]	37	0	0
Missing element			
3 bromide	2	1	0
4 strontium	3	1	0
5 fluoride	46	24	7
6 rubidium	39	30	12
7 iodide	47	34	31
8 zinc	64	36	15
9 cobalt	15	6	1
10 vanadium	34	24	10
11 molybdenum	36	9	5
12 manganese	40	37	27

[1] natural seawater diluted to 12°/ooS.
[2] artificial seawater stock + all trace elements

The results of this trial confirmed the apparent larval requirement for bromide in the artificial seawater (Table XII). There was total mortality in the larvae in treatment 4 (no strontium added). Only in treatment 3 were dead larvae found in any number that could be attributed to the effect of the treatment and not cannibalism.

This experiment was aborted after 96 hours due to several experimental problems. These were:

1) The larvae in tank 2 (control) were lost into the filter, therefore there was only one control.

2) Evaporation caused a daily increase in salinity of 2°/ooS. The fluctuation in salinity caused by the addition of deionised water to correct this was perceived as an additional stress to any other placed on the larvae and an undesirable confusing factor.

3) Although larval mortality occurred in all treatments, the larvae appeared very healthy (except treatments 3 and 4). It was concluded that cannibalism rates were unacceptably high to attribute any significance to the results.

4) Temperatures varied between tanks by several degrees. This was due to the model of aquarium heater used and fluctuations in the air temperature. This was partially due to the small volume of the recirculating aquaria used.

The conclusion drawn from this work was that the ASW stock with all the trace elements except bromide supported larvae, and that the omission of the other trace elements did not have a significant effect. Thus a rearing trial was decided upon to demonstrate whether larvae would survive in ASW with only bromide and strontium added. This would confirm whether the artificial seawater stock and the artemia fed to the larvae contained sufficient trace elements to allow complete larval development.

4.6.4 Experiment 4 - Larval growth and survival in artificial seawater stock (ASW) with bromide, strontium and EDTA.

The aim of this set of trials was to establish whether the artificial formulation devised was comparable to seawater for the rearing to postlarvae of *M. rosenbergii* larvae, and whether the addition of bromide to the artificial seawater was necessary for growth. The rearing media used were as follows:

1) **ASW* + Br + Sr + EDTA** artificial seawater stock (ASW),

	bromide, strontium, EDTA.
2) ASW* + Sr + EDTA	artificial seawater stock (ASW), strontium, EDTA.
3) Seawater*	seawater control.

* Both the ASW treatments and the seawater controls had a salinity of 12 °/oo.

Larval rearing was performed as described in section 3.2 using 70 dm^{-3} black polypropylene rearing bins and 'Eheim' biological filters. No water changes were practised in any of the treatments over the course of the larval rearing period.

Trial 1

In this trial larvae at an advanced stage (VII) were stocked into the rearing media. They had previously been reared in natural seawater diluted to 12°/ooS. No water exchanges were practised during the rearing period and water quality was good throughout this trial. The artemia used in this trial were enriched using 'SELCO'. Larval density at stocking was between 88-91dm^{-3}. The results of the two tanks are summarised in Table XIII.

Table XIII. Larval survival in natural seawater and ASW + Bromide, Strontium and EDTA.

Treatment	DAY in	DAY out	larval stage	larval Nos. in	larval Nos. out	% survival	time
Seawater,12°/ooS	15	31-42	VII	6211	4186	67.4	42 days
ASW,Br,Sr,EDTA	15	30-40	VII	6358	4002	62.9	40 days

Larval numbers

	DAY 15	DAY 18	DAY 20	DAY 42
Seawater, 12°/ooS	6211	5987	5354	4186
ASW,Br,Sr,EDTA	6358	6230	6046	4002

% survival between days

	15-18	18-20	20-42	15-20	18-42	15-42
Seawater, 12°/ooS	96.4	89.4	78.2	86.2	69.9	67.4
ASW,Br,Sr,EDTA	97.9	97.0	66.2	95.1	64.2	62.9

The larval survival in the two rearing tanks is comparable, with the larvae in artificial seawater completing metamorphosis slightly quicker than the larvae reared in natural seawater. Generally, survival between days was comparable for the two tanks. Any differences between the treatments could be due to a number of factors such as the slightly different stocking density, any variations in temperature, feeding rate or cannibalism. These results do show that artificial seawater stock (ASW) can suppport larvae past metamorphosis with only strontium, bromide and EDTA added. Thus all other trace elements that are required by the larvae are either present as impurities in the salts used, or are provided by the artemia. If there is an effect of the medium on the growth of the larvae it does not appear to be substantial.

Trial 2

This trial was performed to observe the effect of lack of added bromide on larvae reared in ASW. There were three treatments in this trial, the intention being to replicate the experiment three times. Tank 1 contained natural seawater at 12°/ooS, Tank 2 contained ASW+bromide, strontium and EDTA and Tank 3 contained ASW + strontium and EDTA. Larvae were stocked at stage three, having been hatched in natural seawater (12°/ooS). No water exchanges were performed in any of the tanks during this trial. Water quality was generally good throughout the trial. The artemia used in this trial were enriched using 'SELCO'. The results of this trial are summarised in Table XIV.

There was total mortality in tank three two days after stocking. The larvae in this treatment rapidly became moribund and when observed in a glass beaker they did not swim actively as did the larvae from the other two tanks. Mortality seemed to occur during or just after moulting. Larvae that were the most affected seemed to be only partially moulted. Dead larvae at stage IV were observed. There was little evidence that the larvae had been feeding, although this is difficult to determine in these small stages. The larvae in the other two treatments grew well, reaching metamorphosis in 27-32 days after hatching. Survival was higher in the natural seawater tank, but time to metamorphosis was quicker in the artificial seawater tank. The lower survival in the artificial seawater might be as a result of increased cannibalism due to the faster growth rate of the larvae in this treatment.

Table XIV. Effect of lack of bromide on larval survival in ASW.

Treatment	DAY in	DAY out	larval stage	larval Nos. in	larval Nos. out	% survival	time
seawater,12°/ooS	3	23-32	III	4733	3046	64.4	32 days
ASW,Br,Sr,EDTA	3	23-27	III	4831	2612	54.1	27 days
ASW,Sr,EDTA	3	5	III	4939	0	0.0	2 days

	Larval numbers DAY				
Treatment	3	14	23	27	32
Seawater,12°/ooS	4733	4721	3093	3060	3046
ASW,Br,Sr,EDTA	4831	4829	3517	2612	-
ASW,Sr,EDTA	4939	-	-	-	-

	% survival between days					
	3-14	14-23	23-27	3-23	14-27	3-27
Seawater, 12°/ooS	99.7	65.5	98.9	65.3	64.8	64.7
ASW,Br,Sr,EDTA	99.9	72.8	74.3	72.8	54.1	54.1
ASW,Sr,EDTA	0	-	-	-	-	-

This trial proves that larvae can be reared successfully past metamorphosis in artificial seawater stock containing bromide, strontium and EDTA. It also appears that the addition of bromide to ASW is critical for the survival of the larvae.

Trial 3

This trial was a repeat of the previous trial with an additional treatment. The additional treatment contained artificial seawater stock, strontium and EDTA and an elevated bromide concentration. This concentration of bromide was 3.375 $mmol.kg^{-1}$, and is equivalent to a concentration of bromide four times that of seawater at 12°/ooS (0.8437

mmol.kg^{-1}). There were consistent problems with water quality during this trial, with nitrite values occasionally exceeding 0.25 mg.dm^{-3} NO_2-N. This was principally due to the very high larval stocking densities employed in this trial (≈156 larvae.dm^{-3}). The artemia enrichment used was the formulation of Watanabe *et al.* (1982) (Appendix II). The results of this trial are summarised in Table XV.

Table XV. Larval survival in natural seawater and artificial seawater media containing varying concentrations of bromide.

Treatment	DAY in	DAY out	larval stage	larval Nos. in	larval Nos. out	% survival	time
seawater, 12°/ooS	2		II	10700	0	0.0	-
ASW,Br,Sr,EDTA	2	33-42	II	10700	2962	27.6	42
ASW,Sr,EDTA	2	6	II	10700	0	0.0	6
ASW,4xBr,Sr,EDTA	2	33-42+	II	10700	4290	40.09	42+

Larval numbers

	DAY 11	DAY 20	DAY 25	DAY 33	DAY 42
seawater,12°/ooS	8329	3963	-	2213	-
ASW,Br,Sr,EDTA	10161	8863	7102	4672	2962
ASW,Sr,EDTA	0	-	-	-	-
ASW,4xBr,Sr,EDTA	13424	10523	8901	6044	4290

% survival between days

	11-20	20-25	25-33	33-42	11-33	20-42	25-42	11-42
seawater, 12°/ooS	47.6	-	-	-	26.6	-	-	-
ASW,Br,Sr,EDTA	87.2	80.1	65.8	63.4	46.0	33.4	41.7	29.2
ASW,Sr,EDTA	-	-	-	-	-	-	-	-
ASW,4xBr,Sr,EDTA	78.4	84.6	67.9	71.0	45.0	40.8	48.2	32.0

The larval survival in this trial was very poor compared with the previous trials. In the natural seawater treatment there was a loss of larvae on day 17 due to the loss of a filter screen in the larval tank. This caused some of the larvae to be sucked into the biological filter where they became trapped and subsequently died. This reoccurred on day

35 resulting in the loss of all the larvae.

The slow rate of development of the larvae and generally poor survival can be attributed to three causes in this trial.The water quality of all the larval tanks was quite variable with occasionally high nitrite concentrations. When this occurred, feeding was suspended, or reduced to allow the reduction of the nitrite. The very high stocking densities meant that large amounts of artemia were fed each day, this placed a strain on the biological filtration and was probably the root cause of the poor water quality. The enrichment compound used for the artemia did not seem to be very satisfactory. Whilst the artemia were seen to contain oil droplets, as did the larvae, the artemia also had high concentrations of bacteria associated with their anal region. This was never observed with the other enrichment compounds. This enrichment was not made fresh every day, and it is possible that deterioration occurred giving rise to high bacterial loadings in the artemia enrichment tank. Interestingly, the artemia also contained faecal material. This was also uncommon when the other enrichment compounds were used. The artemia may have been ingesting the bacteria, and this would explain the bacteria observed around the anal region of the enriched artemia. If the artemia were insufficiently enriched this would result in serious malnutrition of the larvae. This is due to the exhaustion of the carbohydrate and lipid reserves of the artemia during the enrichment process. If these are not replaced by the enrichment compound the nutritional quality of the artemia will be very poor.

4.7 Conclusions

The demonstration of whether an ion is essential to *Macrobrachium rosenbergii* larvae proved to be a difficult task. For the majority of the trace elements impurities in the artificial seawater salt will provide any trace elements required. The artemia that are fed to the larvae will also contain appreciable quantities of trace elements. In order to assess the larval requirement from the medium a highly purified and defined seawater must be used, such as the formulation by Morel *et al.* (1979). This uses analytical grade salts, and relies on ion exchange methods for the removal of trace metals. If the larvae were to be fed, a defined diet would be required. Since this has never been achieved, larval feeding is as yet impossible.

The similar rates of larval survival and development between natural sewater and the artificial seawater used in section 4.6 (ASW + Br + Sr + EDTA) shows that the artificial seawater can be used for larval rearing with confidence. The ability to rear larvae past metamorphosis in both natural and artificial seawater without the need for water exchange shows that the trace element impurities and inputs via the artemia are sufficient to allow normal development. The rate of development to metamorphosis achieved in some trials is further evidence that the depletion of trace elements is not significant over the course of one larval cycle. Whether the seawater can be reused in a recirculation system for repeated larval rearing cycles must be the subject of further research.

This work does show that bromide must be added to the medium in order to allow survival and growth of larval *Macrobrachium rosenbergii.* This is significant since this element has not been shown to be essential (chapter 5, section 5.2). Bromide does not appear to be replacing iodide, since larval survival in the treatment omitting iodide was comparable with the other treatments, and did not result in total mortality as in the treatments omitting bromide. This argument applies to fluoride, where omission of this ion from the rearing medium does not cause total mortality. The following chapter (5. The halogens in invertebrate biology) attempts to clarify the possible reasons why bromide might be essential, and at what concentration it has an effect on the larvae of *Macrobrachium rosenbergii.*

CHAPTER 5 - The halogens in invertebrate biology.

All of the halides are present in the marine environment in decreasing order of concentration Cl>Br>F>I; they are also all present in the tissues of marine organisms. The halides are also found in freshwater organisms although their concentrations are reduced (Vinogradov 1953). That they are conserved in freshwater, even though the environmental concentrations are much lower, is an indication that they are essential for life.

The majority of the research into the role of the halides in marine organisms has focussed on chloride and iodide. The interest in chloride is concerned with its central role in osmoregulation (section 6.0), and its abundance in the blood of marine organisms renders it the most easy halide to analyse. Iodides have been known for a long time to occur in marine algae. The impressive ability of marine algae to concentrate iodide from the environment made them a subject of interest, particularly as the role of iodine in the treatment of goitre made it one of the first trace elements known in man.

The early method of analysis for the halides involved ashing large quantities of material and then chemically analysing the ash. In the case of iodine and bromine the values were for the whole organism. The lack of specificity of the ashing method meant that little was, or indeed is, known about the site of action of these elements and whether they are concentrated in specific parts of the organism. The information regarding the distribution of the halides bromine, iodine, and fluorine, has increased somewhat with regard to distribution in macroalgae, although actual function has not been elucidated. It has been shown that certain marine algae possess cells that produce free iodine, and there are analagous cells that produce free bromine. However, the mechanism by which these halogens are produced is unknown (Roche *et al.* 1963). Interest in halogenated organic compounds from algae is increasing due to the wide range of pharmacological and toxicological effects they possess (Webb 1973).

Distribution of fluoride, bromide and iodide differs between vertebrates and invertebrates. Chloride is found throughout the tissues and body fluids in both due to its role in ionic and osmotic equilibria of tissues. Chloride also has an effect on the rate of

some enzymatic processes, membrane phenomena and the swelling of colloids (Roche *et al.* 1963).

5.1 Fluoride

Fluoride is found in vertebrate blood at a concentration of 2-5 ppm. It does not form part of the structure of any organic molecules according to Roche *et al.* (1963), although Underwood (1977) maintains that plasma fluorine occurs as both ionic and bound forms. The skeleton contains the bulk of the fluorine found in vertebrates; the level in the skeleton usually reflects the dosage received by the animal. The form of fluorine in skeletal structures is fluoroapatite ($3Ca_3(PO_4)_2.nCaF_2$). The major implications as to the role of fluoride in vertebrates is in the correct formation and strengthening of calcareous skeletal structures.

The more recent interest in fluoride in marine organisms was stimulated by Soevik & Braekkan's (1979) discovery of elevated fluoride levels in both North Atlantic and Antarctic krill. The concentrations found (1300-2400 ppm as dry weight) led them to conclude that krill in any form exceeded the safe level set for human consumption. This was considered to be a problem as krill is potentially an abundant source of protein and it was hoped to utilize this for animal feeds, and possibly human consumption.

The study of the anatomical distribution of fluoride within krill revealed that the majority was concentrated in the exoskeleton (Christians *et al.* 1981; Szewielow 1981; Boone & Manthey 1983; Adelung *et al.* 1987). Indeed, Adelung *et al.* (1987) found that over 99 % of the fluoride was located in the exoskeleton (2600 ppm, 3300 ppm dry weight of cuticle, for *Euphausia superba* and *Meganyctiphanes norvegica* respectively), this was in contrast to the low concentration found in the tissues (6 ppm dry weight of muscle). Their study of the incorporation of fluoride into the exoskeleton over the course of the moult cycle in these species revealed that the fluoride is taken up and incorporated at the same time as the cuticle is constructed. This rules out simple adsorption onto the exoskeleton from the environment and suggests a well developed mechanism for the concentration of fluoride from the environment. Hempel & Manthey's (1981) study on the

Antarctic krill (*Euphausia superba*) showed that larval krill had a 50% higher fluoride concentration than the adults. Their conclusion was that the difference was due to a different carapace to body weight ratio between larvae and adults. The form of fluoride in these studies has not been established, as the method of analysis has relied on acid digestion of the samples. This solubilises the fluoride, but may also release any organically bound fluoride. Thus it cannot be determined whether these high fluoride values are due to the hardening of the exoskeleton by fluorapatite, the incorporation of ionic fluoride as other compound are deposited, or the presence of fluorinated organic compounds. The lack of difference in fluoride concentration between different stages of larvae led Hempel & Manthey (1981) to conclude that the larvae had a well developed mechanism for the uptake of fluoride from the environment. If the fluoride were entirely derived from the egg, it would be expected to see a decrease in fluoride with successive larval stages.

In Table XVI the values for the various analyses of fluoride in krill are given. There are many discrepancies, particularly regarding the muscle values. Adelung *et al.* (1987), account for this as contamination of the muscle with carapace material when the samples were taken. Another means by which the fluoride can contaminate the tissue is during storage. Even when frozen there is a gradual migration of fluoride into the tissues (Christians & Leinemann 1980,1983). The fact that the fluoride is so labile suggests that it is present as ionic fluoride; if the fluoride were organically bound it would not be expected to leach into the tissues when frozen.

Fluoride is also found in the hard structures of other marine invertebrates such as corals and molluscs. Vovelle *et al.* (1989) found significant amounts of fluoride associated with the jaws of the polychaete annelid - *Hyalinoecia tubicola* (Muller). The distribution of fluoride was found to be similar to that of calcium and strontium, but not to phosphorous. The lack of phosphorous in the jaws rules out the possibility of the fluorine being present as fluorapatite. The conclusion reached by these authors was that the fluorine was present as CaF_2 and SrF_2 in association with the aragonite ($CaCO_3$) of the jaws, and that its role was the same as in the fluoride hardening of teeth of higher vertebrates. In the freshwater molluscs, the concentration of fluorine found in the shell is less than that of marine forms. This is attributed to the fact that the concentration of fluoride in seawater is approximately ten times that of freshwater (sea, $n \times 10^{-4}$; rivers $n \times 10^{-5}$) (Vinogradov 1953).

Table (XVI) Fluoride concentrations (ppm d.w.) in adult krill - adapted from Adelung *et al.* (1987).

Species	whole krill	exoskeleton	muscle	hepatopancreas	ref.
E. superba	1058	2594	4.5/2.9	-	a
	2400	3330	570	-	b
	1950	-	325	-	c
	1532	9000-14000	80-360	-	d
	-	-	18	-	e
	780	-	60	-	f
	1650	-	-	-	g
	<1000	-	-	-	h
	1009	1958	70	7.6	i
	330*	1200*	44*	-	j
	–	–	145-155	-	k
M. norvegica	2153	3343	5.7/3.0	4.2	a
	1330/2360	-	-	-	b
	2261	-	-	161	l

*) values based on wet weight

a) Adelung *et al.* 1987
b) Soevik & Braekkan 1979
c) Schneppenheim 1980
d) Christians & Leinemann
e) Christians *et al.* 1981
f) Szewielow 1981
g) Ellingsen 1982
h) Hempel & Manthey 1981
i) Boone & Manthey 1983
j) Walters *et al.* 1983
k) Casalta *et al.* 1984
l) Boone 1981

5.2 Bromide

Bromine occurs predominantly as bromide in the marine environment and also in living organisms (Table XVII). The bromide found in the tissue fluids of animals is found as protein salts and is dialysable; it does not seem to form organic compounds as does iodine (Roche *et al.* 1963). The fate of bromide in vertebrates seems to be linked with that of chloride as the serum concentration can be influenced by selective administration of one or the other. Indeed, the treatment of bromide poisoning in humans is the administration of

massive doses of chloride. There does not seem to be a selective mechanism for the excretion of bromide. However, there is reason to believe that a proportion of the bromide determined in some living organisms is present as organically bound bromine. The total amount of bromine on the earth is estimated at 10^{16} tons of which about half is thought to be contained in living organisms (Jolles 1966).

Table (XVII) Relative concentrations of bromide in rock, water, marine, and terrestrial organisms (in %) - from Vinogradov (1953).

	Terrestrial	Marine
Air	$n \times 10^{-6}$	$n \times 10^{-5}$
Water	2×10^{-6}	6×10^{-3}
Rock	$n \times 10^{-4}$	-
Soil and silt	$n \times 10^{-3}$	$n \times 10^{-3}$
Plants	$n \times 10^{-4}$	$> 10^{-3}$
Animals	$n \times 10^{-4}$	$n \times 10^{-3}$

The precise physiological role of bromine/bromide has not been satisfactorily defined as yet, although experimental evidence has shown that it may be essential for vertebrates. Underwood (1977) mentions two studies that attempted to show the essentiality of bromine. Although neither was conclusive, he comments that the growth requirement for bromine has not been pursued further. Isolator experiments have not been performed as yet. The main reason for the lack of research into the essentiality of bromide is probably due to its ubiquitous presence in living organisms. This would make deficiency diseases unlikely and hence the site or mode of action would not be revealed. Bromides were used in human medicine as early as 1835, nine years after the discovery of bromine by Balard in 1826, although their effect on the nervous system was not discovered until 1850 (Jolles 1966). Soon after this they were used in the treatment of epilepsy and as mild sedatives. Bromides exert a prolonged depressant action on all human cerebrospinal centres with the exception of those in the medulla. In therapeutic dosages mild sedation is achieved, excessive dosages can cause confusion, delirium, stupor and even coma (Jolles 1966). Jolles (1966) continues by stating:

> *"The mechanisms by which bromide depresses nervous activity have still to be elucidated. Since tissue cells with the exception of erythrocytes are relatively impermeable to chloride and bromide, it may be that nerve cells are particularly sensitive to the*

extracellular chloride/bromide ratio; on the other hand brain cells do contain some diffusible chloride and it is not certain that bromide remains entirely extracellular. A fall in blood chloride not associated with bromide administration does not bring about central nervous depression; the bromide ion itself is believed to be the active agent."

Bromine has been implicated in the detoxification of mercury in California sea-lions (Martin *et al.* 1976). In a study of the livers of these animals significant, correlations were found between low bromine in the livers and premature pups. Their conclusion was that the low levels of bromine found in the mothers giving birth to premature pups, had the effect of not protecting the pups against either the high internal selenium or mercury concentrations that they all had. However, the authors mention that other factors may have been involved such as the incidence of Leptospirosis or high PCB concentrations in some of the prematurely parturient mothers.

The occurrence of bromine in invertebrates has been studied by a number of authors. This is due primarily to the discovery of large concentrations of organically bound bromine in certain anthozoans (Low 1949,1950). Friedlander (1909) was the first person to elucidate the structure of the dye Tyrian purple. This is extracted as a colourless precursor, which upon exposure to light breaks down to form 6,6-dibromoindigo. The colourless precursor is extracted from gastropod molluscs of the genus *Murex*. The role of this compound in the physiology of these molluscs is unknown. Studies of other organic bromine compounds have been more rewarding. These have shown the presence of brominated tyrosines as a component of scleroproteins in several phyla (a list is given in Table XVIII). Scleroprotein is a protein that has been 'tanned' by the oxidation of diphenols to quinones (section 7.2). It is thought that as a result of the action of oxidase enzymes on the quinones, the bromides and chlorides, and to a lesser extent iodides, present are oxidised to the free halogen. In this form it then readily substitutes into the tyrosine groups that are present in the protein. It is also possible that the quinones themselves might oxidise the halide to the free halogen (Hunt 1984). The evidence for this incorporation of the halogens into scleroproteins being a secondary consequence, is given as the lack of iodinated and brominated scleroproteins in the freshwater and terrestrial representatives of the species studied; these contain only chlorinated scleroproteins (Andersen 1972; Hunt unpubl. 1982 in Hunt 1984). If the incorporation of the halogens into the scleroprotein was internally mediated by a transport process then it would perhaps be expected that brominated and iodinated

scleroproteins would be found in freshwater forms. Hunt (1984) concludes that the concept of a nonspecific mechanism is not wholly satisfactory as in some scleroproteins constant ratios are found between bromo and chloro derivatives. His explanation is that this may be a "function of protein conformation and tyrosyl environments". Thus this author considers that the presence of halogenated scleroproteins a secondary post-translational modification of proteins, caused by the action of enzymes on secondary metabolites.

The reason why iodinated and brominated scleroproteins are found in the marine environment can be explained by considering the relative ease of oxidation of the halogens. The abundance of the halogens is in order of decreasing concentration Cl>Br>F>I, and the oxidation potential, in order of decreasing ease of oxidation I>Br>Cl>F. Although this can explain why bromo and iodo compounds exist in preference to chloro compounds, it still does not seem to explain the large amount of iodine found in the Anthozoa. Low (1949) studied the bromine and iodine content of 46 species of sponge and found a mean ratio of 1.0, this implies a vastly greater concentration of iodine than bromine, as the ratio of bromide to iodide in seawater is approximately $1 : n \times 10^{-4}$. Table XVIII taken from Hunt (1984) lists the halogenated amino acids so far identified in animal proteins.

Table (XVI) **The various halogenated amino acids isolated from animals. Taken from Hunt (1984).**

Amino acid	Sources
3-chlorotyrosine	cuticles *Schistocerca gregaria* (Insecta) *Limulus polyphemus* (Arachnida)(sic) operculae *Buccinum undatum* (Mollusca) *Viviparus viviparus* (Mollusca)
3-bromotyrosine	scleroproteins Porifera (as spongin). Coelenterata (as gorgonin and antipathin) Molluscan operculum cuticles *Cancer pagurus* (Crustacea) *Limulus polyphemus*
3-iodotyrosine	Widely in scleroproteins of In the endostyle and tunic in Ascidiacea (tunicates) and other members of the deuterostome line of invertebrates. Invertebrate thyroglobulins. In *Planorbis corneus* (Mollusca). In *Periplaneta americana* and other insect proteins. In *Nereis divesicolor* (Annelida) scleroprotein
3-iodotyrosine	In byssus and periostracum of *Mytilus galloprovincialis*
3,5,-dichlorotyrosine	Molluscan operculum. *Limulus polyphemus* cuticle.
3,5,dibromotyrosine	Molluscan operculum. *Limulus polyphemus* cuticle. *Cancer pagurus* cuticle. In scleroproteins of sponges and coelenterates.
3,5-diiodotyrosine	Distribution similar to that of 3-monoiodotyrosine.
3,3'-diiodothyronine	In thyroglobulins and thyroid tissue of vertebrates

Amino acid	Sources
3,5,3'-triiodothyronine	In coelenterate scleroproteins (traces). In *Ciona intestinalis* (Ascidiacea). In nemertean mucus. In thyroglobulins.
3,3,5'-triiodothyronine (T_3)	In thyroglobulins.
3,5,3',5'-tetraiodothyronine (thyroxine T_4)	In coelenterate scleroproteins (traces). In deuterostome invertebrates. In insect muscle. In thyroglobulins.
3-bromodityrosine	*Cancer pagurus* cuticle. *Cancer pagurus* cuticle
3-bromotrityrosine	*Cancer pagurus* cuticle
6-bromotryptophan	*Mytilus edulis* (Mollusca) periostracum.
2-monoiodotryptophan	In vertebrate thyroglobulins. Possibly in insects.

Winkler (1969) studying the mollusc *Aplysia californica* found that 90% of the organic bromine compounds were present in the digestive gland, and the rest were confined to the foot and body wall. In the blood, organic compounds were practically absent. The presence of these compounds in the body wall and foot is unsurprising since these are tissues rich in scleroprotein. However, the presence of such large concentrations in the digestive gland suggests some other function. It is possible that these organic bromine compounds have their origin in the algae on which these animals feed.

Several other studies have found bromine to be present in arthropods although these were whole body analyses and failed to show the location of the bromine. Frankel & Jellinek (1927b) reported the presence of iodine and bromine in *Limulus polyphemus*. They found only a little phosphate and no calcium carbonate. The very low ash value of *Limulus* cuticle suggests that it is composed predominantly of chitin and scleroprotein. Vinogradov (1953) found calcium in *Limulus* cuticle, but no evidence of apatite or other crystalline phosphate. This species is of interest as it is assumed to be ancestral to the spiders, and indeed the cuticle is very similar in its composition.

Vinogradov (1953) reported that data on the bromine content of crustacea were unavailable apart from those studies performed in his laboratory. Table XIX summarises his findings and those of Spaargaren (1988).

Table (XIX) Concentration of bromide/bromine in whole analyses of crustaceans.

Species	ppm wet weight	reference
Calanus finmarchicus	100	Vinogradov 1953
	74	"
Gammarus locusta	562	"
Hyas araneus	250	"
Eupagurus palescens	180	"
Pandalus borealis	107	"
Crangon crangon	118	Spaargaren (1988)

The study by Vovelle *et al.* (1983) into the formation and composition of the setae of the polychaete *Petta pusilla* (Malmgren), yielded some very interesting results concerning the possible relationship between sclerotised and calcified tissue. They found that young setae were low in calcium but rich in proteins, but that the opposite was true for old setae. The external face of the setae was reinforced by protein, the internal face by calcium. In individual setae there was a very unequal partitioning of the iodine and bromine. Iodine tended to be concentrated towards the top of the setae, bromine in the bottom. The concentration of each halogen also varied between setae. Young setae were rich in bromine at the base and low in iodine. Chlorine was also detected but at a constant, low level. This underlines Hunt's (1984) hypothesis of the relative non-specificity of the process of halogenation. Vovelle *et al.* (1983) conclude that the halogenation of the tyrosine groups in the protein of the setae assists in the quinone tanning process by rendering them more reactive. Thus there appears to be two possible reasons for the presence of halogens. One is that they are a side effect of the tanning process, the other is that their presence assists the tanning process. What is not clear is why the halogen constitution changes as the setae grow. Does this result from a gradual displacement of bromine by iodine from the environment, or is the change physiologically controlled?

The question that has not been addressed is whether halogenation of the

tyrosine groups in a protein modifies its chemical or physical properties. If the physical properties are modified in some way, for example elasticity, then it may be desirable to have a rigid section and a flexible section in the setae. This is apparent in part in the setae of *Petta pusilla,* where one face is calcified and possibly more rigid than the other, which is predominantly composed of sclerotised protein. Hunt & Breuer (1971) concludes that the presence of halogens in the relatively apolar proteins might increase their surface energy, and hence their wettability. This in turn might improve adhesion between structural protein sheets or fibres and hence increase stability. This stability might facilitate the crosslinking process by allowing closer interaction of reactive groups on adjacent proteins.

Spaargaren (1988) studied the effect of low temperature on halide concentration in the prawn *Crangon crangon* and came to the conclusion that bromide was regulated in the same manner as chloride, and that its function in the animals' metabolism was probably osmoregulatory. This work used whole homogenised animals, this means that any contribution from the carapace or gut is not excluded. Thus, in showing the response to increasing salinity the values obtained are the osmoregulatory effect coupled with any bromide from tissues high in bromide. It may be that his assumption was that organically bound bromine would not be detected (he used an ion selective electrode). If this is the case then the haemolymph concentration of bromide is extremely high indeed, double that of seawater. If bromide were being osmoregulated in the same manner as chloride it would be expected that the concentration in the blood would more or less reflect the environmental ratio between bromide and chloride. This may still be so, in which case there is a significant extra contribution of bromide from another source.

Dunson (1984) studying the permeability of the chitin covered gills of the horseshoe crab *Limulus polyphemus* found they were very permeable to water. However, the permeability of the gills to sodium and bromide was 1000-14000 times less than that of their permeability to water. His explanation for this is that these ions are associated with water molecules and the combined size renders them too large to pass through the pores in the chitin matrix. Dunson also comments that the integument of *Limulus polyphemus* is far less permeable than the gills. This must be due in part to the additional thickness of the integument, but might also be due to sclerotisation, and impregnation with waxes.

5.3 Iodine

Iodine is perhaps the best studied of the halogens due to the early discovery of its essentiality. Even though it has been studied extensively, its mode of action in the body is not known. In vertebrates iodine is concentrated mainly in the thyroid and is present as a variety of compounds. These are given in the last section (Table XIX). Due to the wide range of effects caused by iodine deficiency and thyroid dysfunction the manner by which the iodine containing compounds in the body are thought to act is by an effect at the nuclear level (Rheinhold 1975).

The iodine concentrations in marine algae, marine invertebrates and fish are reviewed in depth in Vinogradov (1953). Seawater iodine concentrations can vary greatly and, as a result the concentrations found in organisms in different areas varies accordingly. It is assumed that the role of iodine is more or less the same for all vertebrates. However, its role in invertebrates is unknown, although iodinated tyrosine compounds are found in scleroproteins. If iodine had a role in invertebrate physiology similar to that in vertebrates, then it could be expected to be found in the body fluids. This would be especially so in the arthropods, since their circulatory systems are better separated from the environment than those of the molluscs. If this were the case then the animals would have to take this up from their food, or from the environment. It is therefore possible that this could be a limiting condition should ambient seawater levels be low.

Vinogradov's (1953) analysis of the copepod *Calanus finmarchicus* (Table XX) is particularly interesting in the light of his statement:

> *"In general, the amount of iodine in diatoms is approximately the same as in the Phaeophycae, which are rich in the element. Thus diatoms play an important role in the migration of iodine in the sea. In the nutrition of many plankton feeding animals this is of great physiological importance."*

This statement implies that the environmental concentration of iodine might be limiting to some higher marine organisms, and that concentration up a food chain is the most effective way it is obtained by those organisms. The primary food items for many larval fish are copepods and these feed on algae, thus the high iodine and bromine

concentrations found in these and other crustaceans might be an important dietary source of these elements, particularly iodine. The form of the halides in these diatoms is not given, but it is possible that they are present as both ionic and organically bound forms, as found in macroalgae. Vinogradov (1953) also gives an iodine concentration of 30 ppm as dry weight for his analysis of a group of diatoms, *Skeletonema, Chaetoceros,* and various *Thalassiothrix.* These are all species which have been found to be extremely valuable/essential in the culture of larval marine species.

Table (XX) The composition of *Calanus finmarchicus*, adapted from Vinogradov (1953).

ELEMENT	WET WEIGHT ($mmol.kg^{-1}$)	DRY WEIGHT* ($mmol.kg^{-1}$)
O	49993.7	15828.1
H	102600.0	732175.0
C	508.3	36309.5
N	1085.7	7755.1
Cl	295.8	2112.7
Na	234.8	1677.0
K	74.2	529.9
S	43.7	311.9
P	42.0	299.8
Ca	10.0	71.4
Mg	12.3	88.2
Fe	1.3	9.0
Si	2.49	17.8
Br	1.13	8.0
I	0.0157	0.110

*). Dry weight values calculated using the value of 86% water content for *Calanus finmarchicus.* This value is given in Vinogradov (1953).

Vinogradov (1953) lists the results of many analyses of iodine in marine organisms and whilst the levels found are higher than for freshwater and terrestrial organisms they are not inordinately so. There is a problem in comparing vertebrates with invertebrates in this respect, due to the localisation of iodine in the vertebrate thyroid. The only study on halide levels in a prawn exposed to different temperatures and salinities has been conducted by Spaargaren (1988) who found that over a salinity range of 5-37°/oo, the iodine concentration in whole *Crangon crangon* fluctuated between

0.05-0.1 mmol.kg^{-1} (6.35-12.69 ppm). The fluctuation did not appear to be caused by salinity since prawns in the lowest salinities had the same level of iodine as those in high salinity. The concentration of iodine in this species is much higher than the environmental level of 0.0004-0.0006 mmol.dm^{-3} (0.05-0.078 ppm). Whether this is stored, or is present as free iodide in the blood is unknown, as is whether some of this iodide is incorporated into other structures. Spaargaren (1988) found no appreciable difference between male, and ovigerous and non-ovigerous female *Crangon crangon*. However, the standard deviations of his results were enormous and as such cannot be considered to be extremely reliable. Closs (1931) (in Vinogradov 1953) found iodine concentrated in the ovary, but not the testes of the lobster *Homarus gammarus* at concentrations of 0.796 and 0.0362 mmol.dm^{-3} (101 and 4.6 ppm) respectively.

The vertebrate growth hormone thyroxine contains iodine, and its effect on the growth and moulting of *Penaeus monodon* postlarvae and juveniles was studied by Pillai *et al.* (1987). They found that significant increases in growth rate were obtained using 3.0 µg.dm^{-3} (postlarvae) and 5.0 µg.dm^{-3} (juveniles). The effect of a vertebrate growth hormone on a crustacean is intriguing as it would seem that the increase in iodine concentration represented by the treatment was comparatively small (approximately 2.6-3%); this rules out the effect of iodine being supplemented and suggests that the thyroxine itself was having an effect. However, it may be that thyroxine is a particularly easy form of iodine to assimilate and thus the actual increase in iodine concentration, though small, could be significant.

The comparison of marine and freshwater species of the same genera will perhaps be the most rewarding method of establishing the different roles of the halides in the physiology of animals. The study of anadromous and catadromous species should also prove to be of interest, as their movement between the two environments exposes them to very different levels of all the halides. In this respect *Macrobrachium rosenbergii* is an ideal species to study, since it is closely related to the brackishwater palaemonid species, and yet the adults are adapted to life in freshwater. Its ability to move between fresh and brackishwater also allows its response to changing environmental ionic concentrations to be studied.

5.4 Effect of bromide on larval survival and growth.

5.4.1 Experiment 1

The apparent requirement for environmental bromide by larval *Macrobrachium rosenbergii* was demonstrated in section 4.6. The aim of this experiment was to test whether that environmental requirement had a threshold level. That is, was there an environmental bromide concentration below which larval mortality occurred, but above which survival was unaffected? Determination of this level, and comparison with adult bromide regulation data, might also indicate whether the larvae are able to actively concentrate the bromide from the medium, or whether uptake is a relatively passive process.

Materials and methods

Stage I larvae, hatched in freshwater were used for this experiment. This was in order that there would only be the maternally invested bromide available to the larvae. The larvae did not change stage in the freshwater they hatched in, but did so once introduced to the test media.

Larvae were placed in 500 cm^3 'Pyrex' beakers containing artificial seawater stock (section 4.5), and a range of bromide concentrations. The bromide concentrations used were; 0, 0.15, 0.30, 0.45, 0.60, 0.75, 0.90 $mmol.dm^{-3}$ (added as KBr). These concentrations correspond to the bromide concentrations found in seawater of salinity 0-35 $^{o}/oo$. There were 30 larvae per beaker and three replicates per treatment. The beakers were held at $27^{o}c$ in a water bath, and the beakers were not aerated. The larval density was deemed insufficient to warrant aeration, as the effect of aeration often causes the larvae to become stranded on the sides of the beakers. The larvae were unfed in these treatments.

Larvae were considered to be dead if they appeared torpid on the bottom of the beaker, of if they changed colour to pink or white. When the larvae moult they usually sink to the bottom of the beaker, and it is in this state that they can be mistaken for

moribund animals. The larvae in the treatment containing no additional bromide were obviously dead due to their change of colour.

Results and discussion (Table XXI, Fig 3).

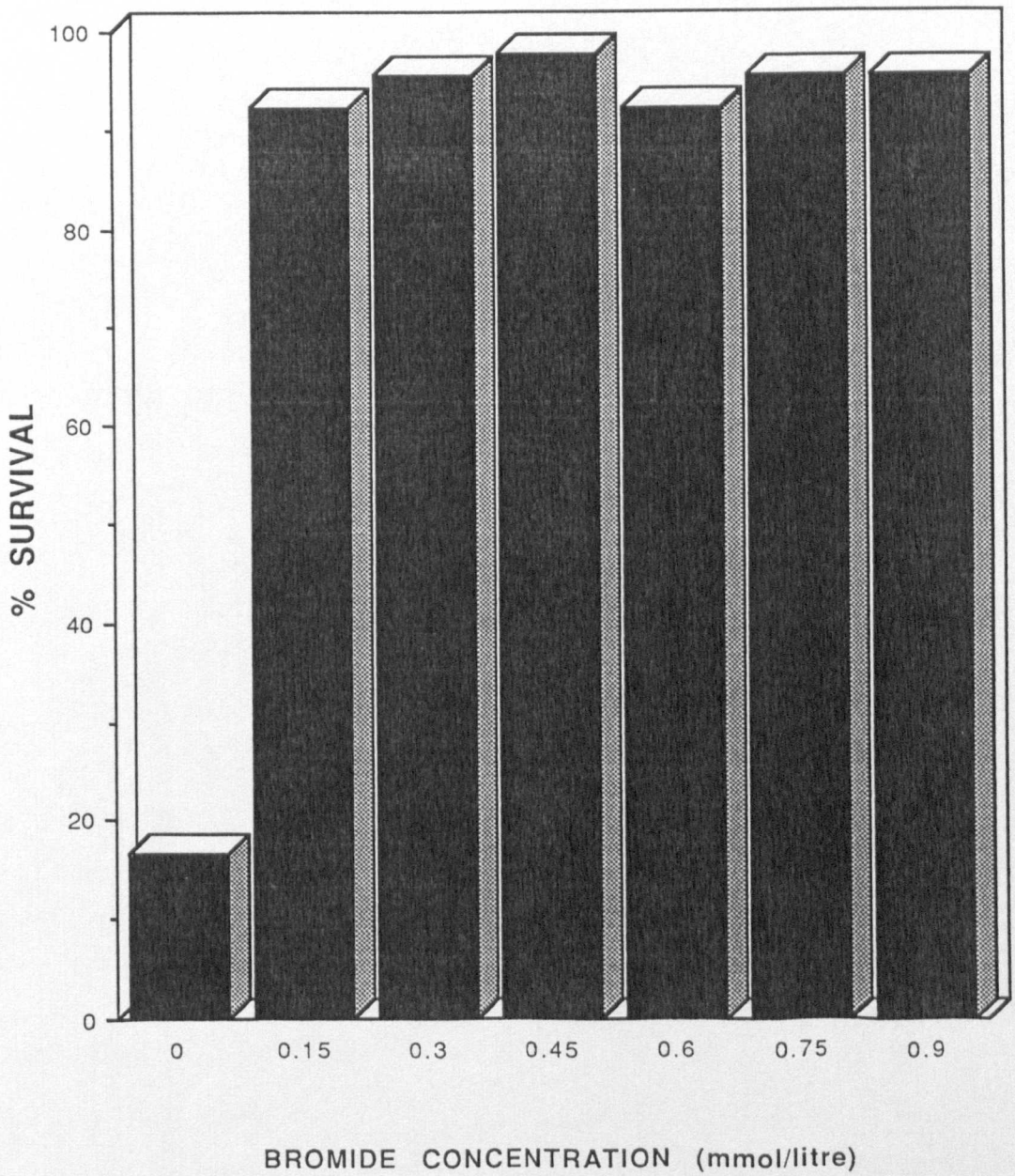
The bromide treatment that yielded the lowest survival was the artificial seawater with no additional bromide. That any survived at all was surprising, but might be attributed to the larval stage used in this trial. The change in form between a stage I and a stage II larva is only the development of eyestalks. This does not seem to require a whole body moult, and as such it might not be particularly traumatic to the larvae. The moult between stages II and III involves the shedding of the whole carapace, and most of those larvae found dead in treatment 1 were in the process of moulting to stage III. Survival in all other treatments was good (92.2-97.8%) with some stage III larvae evident. The larval yolk reserve seems to be sufficient to allow moulting to stage III.

Table (XXI) The effect of bromide concentration (0-0.9 mmol.dm^{-3}) on survival of stage I larvae in artificial seawater over three days. (Results as mean ± s. d.,n=30)

	Added [bromide] mmol.dm^{-3}	Survival % replicate No. 1	2	3	% mean
1	0.00	13.3	23.3	13.3	16.6 ±4.7
2	0.15	93.3	100.0	83.3	92.2 ±6.9
3	0.30	86.7	100.0	100.0	95.6 ±6.3
4	0.45	93.3	100.0	100.0	97.8 ±3.2
5	0.60	90.0	90.0	96.7	92.2 ±3.2
6	0.75	90.0	96.7	100.0	95.6 ±4.2
7	0.90	93.3	100.0	93.3	95.5 ±3.2

The survival of some larvae in the artificial seawater with no added

(Fig.3) The percentage survival of stage I larvae in artificial seawater containing varying bromide concentrations.



bromide might be explained by the use of technical grade sodium chloride in its composition. It is possible that sufficient bromide is present as impurities to allow the larvae to survive for some time. An alternative explanation is that the change from stage I to stage II larvae does not involve the shedding of the cuticle, and hence a significant permeability change allowing the loss of bromide does not occur. All of the larvae that had moulted to stage III were found to be dead. The bromide concentration in treatment 2 is equivalent to the bromide concentration found in 6 $^{o}/_{oo}$ seawater, and gave very good survival. Larval *Macrobrachium rosenbergii* are known to tolerate this salinity of seawater without adverse effects. Thus the threshold bromide concentration that results in larval mortality is between 0 and 0.15 mmol.dm^{-3}. The haemolymph bromide concentration for adult prawns in freshwater was found to be 0.185 ± 0.025 mmol.dm^{-3} (section 6.2.2.5). It would not be expected to find the larval haemolymph concentration below this level, and thus the mortality of the larvae at lower bromide concentrations could be due to permeability changes and loss of bromide. Mortality was not due to salinity shock, as all the other ionic constituents of the medium were present at the concentrations found in 12 $^{o}/_{oo}$ seawater.

5.4.2 Experiment 2.

The aim of this trial was to establish whether the addition of bromide to artificial seawater stock was essential to stage V *Macrobrachium rosenbergii* larvae. Because the threshold concentration that it is necessary to add lies between 0 - 0.15 mmol.dm^{-3}, the concentrations of bromide added to the artificial seawater were considerably lower in this trial.

Materials and methods

Twenty stage V *Macrobrachium rosenbergii* larvae were placed in each of 18 500cm^3 'Pyrex' beakers containing 12$^{o}/_{oo}$ artificial seawater stock (ASW). To this was added Na_2EDTA, and strontium at the concentration normally found in 12$^{o}/_{oo}$ seawater (section 4.5). General purpose grade NaCl was used in the artificial seawater in this trial. Bromide (as KBr) was added to all except three beakers at concentrations of 0.0099, 0.0198, 0.0296, 0.0395, 0.0494 mmol.dm^{-3}. There were three replicates at each

concentration. The highest concentration was equivalent to the bromide concentration found in 2°/oo seawater. The larvae were fed once at the beginning of the trial and aeration was provided due to the presence of artemia. The larvae were counted at the end of the trial three days after stocking.

Results and discussion (Table XXII, Fig 4).

Larval survival in this trial was comparable with that observed in the previous trial (90.0-96.7%) except for the treatment containing no added bromide. In this treatment larval survival was higher than in the equivalent treatment of the previous experiment. There are several possible reasons for this higher survival; the larvae in this trial were fed artemia, and this contributed to the organic loading of the water, this meant that the trial was run for a shorter time (three days instead of four). The later stage of larvae used in this resulted in a greater tnterval between moults , and since this appears to be the time when the lack of bromide has the greatest effect, the short duration of this trial provided the larvae with a better chance of surviving for three days. Cannibalism did not appear to be a problem in this trial. Larvae in the treatment containing no additional bromide appeared somewhat 'wrinkled' relative to the larvae in the other treatments. This is consistent with other observations of larvae in bromide-free artificial seawater made during this work.

The results of this trial clearly indicate that addition of some bromide to artificial seawater appears to be necessary, although the concentration that is required is uncertain. If a trace amount is added, equivalent to that found in 0.4°/oo seawater, it is sufficient to allow larval survival for several days. Whether the larvae are able to extract the low concentration of bromide from the water and survive perfectly well, or whether the trace amount of bromide is sufficient to allow short term survival is unknown. Adult prawns are able to maintain their haemolymph bromide at quite high concentrations when they are in freshwater, and thus appear to possess some mechanism for its homeostatic control (section 6.2.2.5). It is possible that the larvae possess a similar mechanism but its efficiency or their size (i.e. large surface to volume ratio) necessitates the presence of a minimum environmental bromide concentration.

(Fig.4) The percentage survival of stage V larvae in artificial seawater containing varying bromide concentrations.

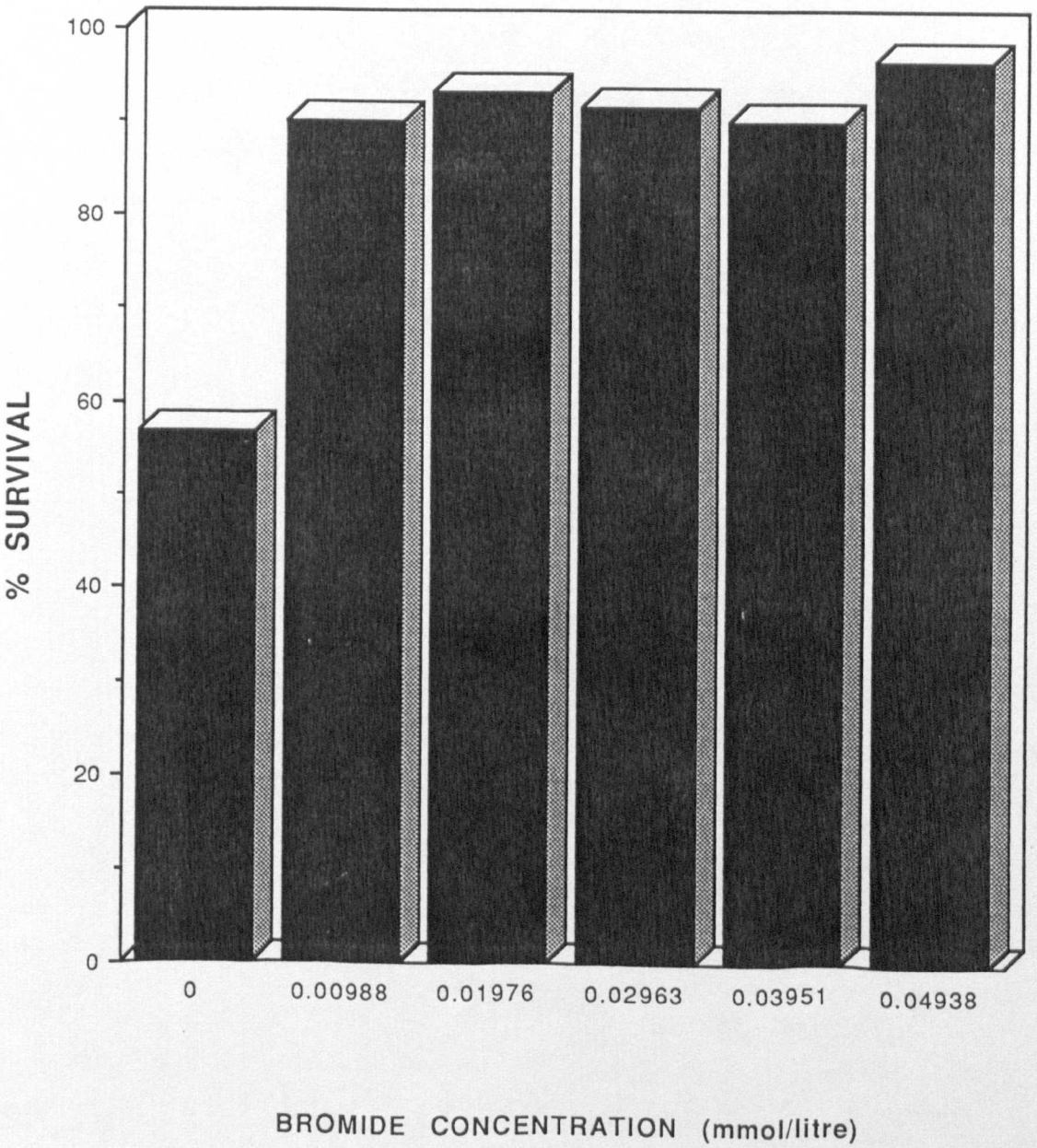


Table (XXII) The effect of bromide concentration (0-0.0494 mmol.dm^{-3}) on the survival of stage V larvae in artificial seawater over four days. (Results as mean ± st. dev.,n=20).

	added [bromide] mmol.dm^{-3}	Survival % replicate No. 1	2	3	% mean
1	0.00000	60.0	60.0	50.0	56.7 ±4.7
2	0.00988	80.0	100.0	90.0	90.0 ±8.2
3	0.01976	85.0	100.0	95.0	93.3 ±6.2
4	0.02963	95.0	85.0	95.0	91.7 ±4.7
5	0.03951	95.0	85.0	95.0	90.0 ±4.7
6	0.04939	90.0	100.0	100.0	96.7 ±4.7

The observation that the mortality seems to occur during, or just after moulting suggests two possible effects of the lack of bromide. It is possible that the increased permeability of the larvae, coupled with their high surface to volume ratio causes an irreversible loss of bromide, and that this bromide is essential for some physiological function. A second explanation is that the bromide has some function in the moulting process. This is suggested by its presence in scleroproteins in crustacean cuticles (section 5.2), and high concentrations of bromide have been found in postlarval cuticles of *Macrobrachium rosenbergii* held in freshwater (present study, section 7.5.6). The deformity of the larvae that moulted in the artificial seawater lacking additional bromide suggests that the cuticle formation process may be affected by the lack of bromide. More specifically, the sclerotisation process might not be occurring normally. This assumes some role for bromide as a cofactor for an enzyme system, or as molecular bromine in organic compounds facilitating the formation of scleroproteins. This last role was suggested by Hunt (1984) as a possible reason for the occurrence of bromine in scleroproteins.

5.4.3 Experiment 3

The aim of this experiment was to try to demonstrate a pharmacological effect of bromide on *Macrobrachium rosenbergii* larvae in the presence of an elevated environmental bromide concentration (see section 4.2). The possible effects of the high bromide concentration were unknown, although previous larval rearing trials using artificial seawater had produced darker larvae than those reared in natural seawater. The growth and survival of the larvae are the parameters that are most easily monitored during rearing trials. A pharmacological effect of bromide could be manifested as an elevated rate of survival, a faster development to metamorphosis or both. The general condition of the larvae in the treatments can give an indication of the effect of the treatment as well.

Materials and methods

There were two experimental treatments and two controls. Control treatments consisted of natural seawater diluted to 12 °/ooS. Experimental treatments contained natural seawater diluted to 12 °/ooS, to which had been added 1.184 mmol.dm^{-3} KBr. This gave an added bromide concentration four times that of seawater at 12 °/oo, making the total bromide concentration five times that of 12 °/ooS seawater (1.480 mmol.dm^{-3}). This bromide concentration is in excess (1.7 times) of that found in full strength (35 °/ooS) seawater (0.8642 mmol.dm^{-3}), and is unlikely to occur in nature due to the relatively stable concentration of this element in seawater (section 4.1).

By the use of natural seawater supplemented with bromide, the problems of water quality deterioration and water loss during rearing were removed. Water exchanges were practised in these trials, although recirculation was also employed. This gave extremely good water conditions, a necessity in the study of potentially small variations in growth performance.

The settlement of filters, tank maintenance and feeding, was as described in section 3.2. An exception to this was the use of a homemade artemia enrichment compound throughout the trial (Appendix 2). This was due to the lack of availability of SELCO. The low stocking densities employed in these trials was an attempt to minimise water quality

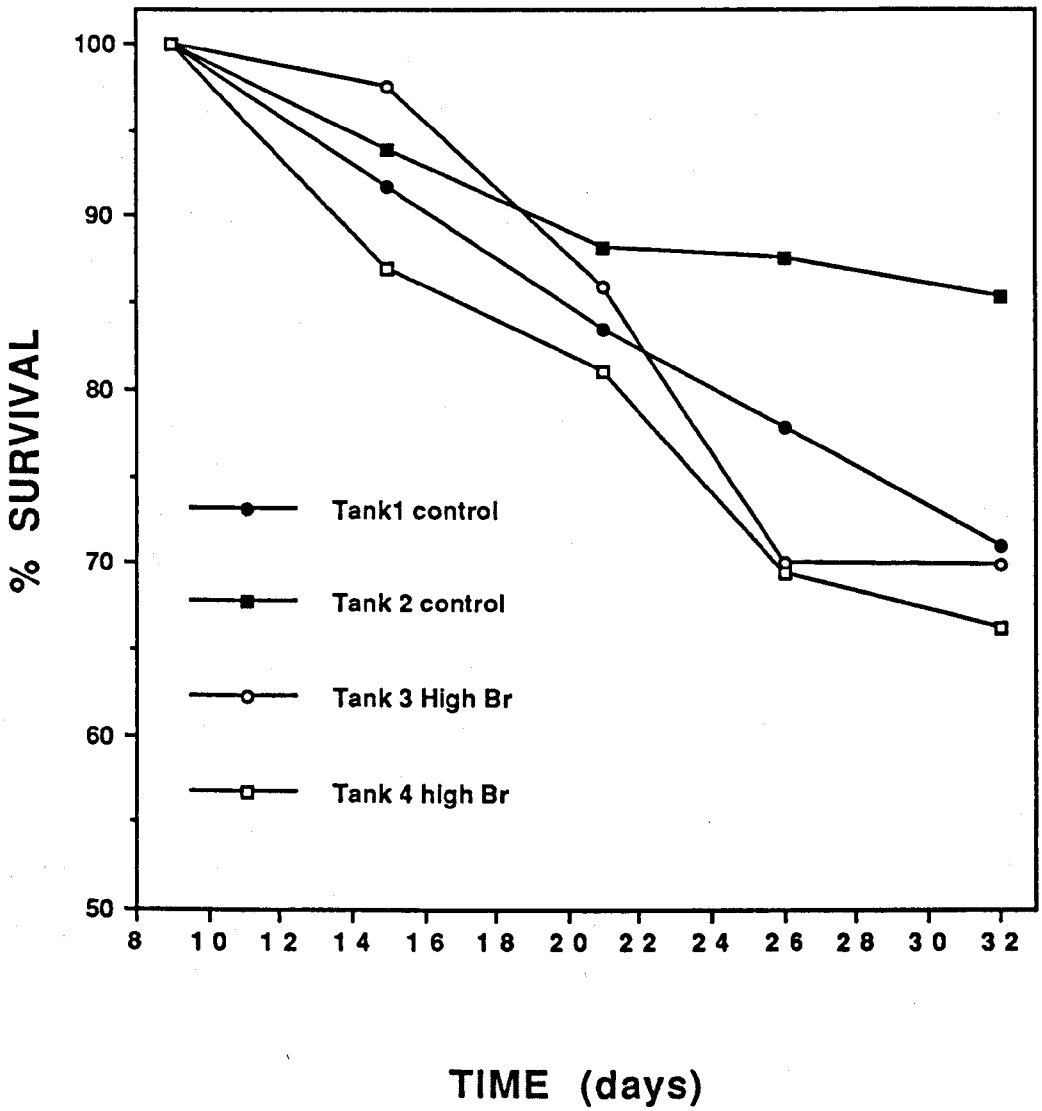
disturbances and cannibalistic interactions. A low stocking density also allowed the provision of enough artemia to ensure that food availability was not a controlling factor in the experiment. Larvae were maintained at 28°C but, due to a problem with the aquarium heaters, the water in the rearing tanks did not remain constant throughout the trial, or between the treatments. The significance of this will be discussed.

Results and discussion (Figs. 5,6,7)

Larval growth and survival were excellent in this trial (Fig. 5). The first postlarvae appeared after 21days of rearing and all of the larvae had metamorphosed by day 32. There was little apparent difference in the appearance of the larvae between the control and experimental treatments, both sets being normally formed with respect to appendages and setation. In this experiment, as in the previous one, the larvae appeared slightly darker in the experimental treatments. When these larvae were examined under a binocular microscope they seemed to have a darker tan coloured cuticle. This tan colouration is consistent with the descriptions of the appearance of sclerotised cuticles described in section 7.2.1. No difference in the patterns of the chromatophores on the larvae was apparent between control and experimental treatments. However, the red abdominal chromatophore may have been slightly more dilated in some larvae from the experimental treatment, adding to their darkened appearance. Pale larvae were observed in both treatments, and it was concluded that these had just moulted. Similarly, dark larvae were observed occasionally in the control treatments, thus the significance of the colouration is uncertain.

Even though care was taken to ensure the same number of larvae were stocked into each culture tank, when the larvae were first counted the numbers in each tank varied widely. This difference in stocking density means that only percentage survival could be used as an indicator of any differences between the treatments. Even the use of percentage survival is not satisfactory since the difference in stocking density means that larval interaction would have been different between the treatments. This might have had an effect on the level of cannibalism even though excess food was supplied. The success of the homemade enrichment compound was demonstrated in this trial with larval survival being comparable or better than in rearing trials using 'SELCO' and 'SUPER SELCO'.

(Fig.5) Percentage survival of prawn larvae in control and high bromide treatments.

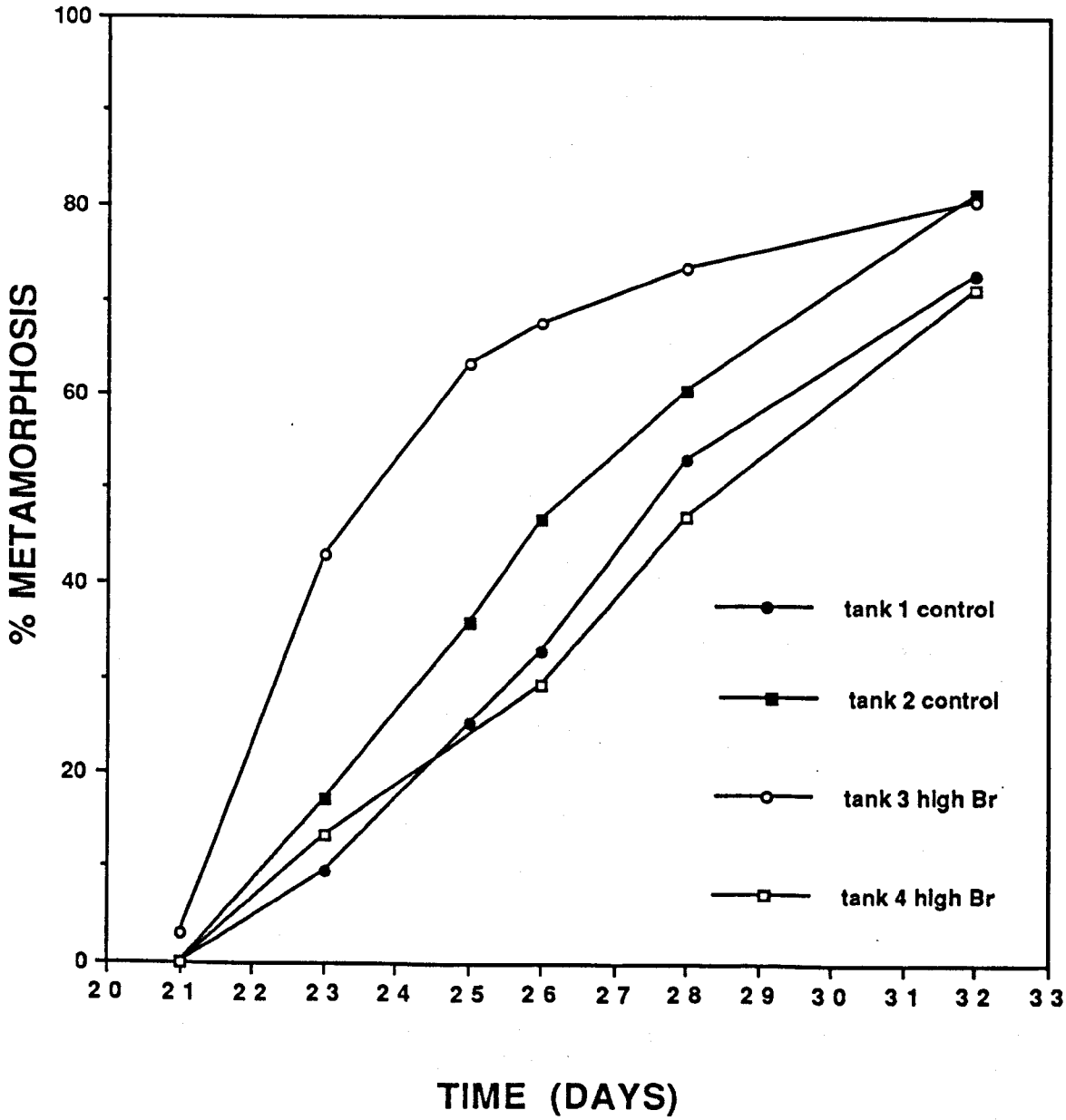


The fact that metamorphosis of the larvae commenced and finished at approximately the same time in all the treatments shows that the addition of excess bromide to the rearing medium does not have a great effect on larval development rate relative to the effect of other factors. The differences found in the rate of metamorphosis (Figs. 6, 7) were possibly due to effect of temperature rather than the effect of the additional bromide. It was found that for the first ten days of rearing the temperature in tank 3 was 28.6°C, tanks 1 and 2 were 28°C, and tank 4 was 27.8°C These temperatures were adjusted and were maintained in the range 27.9-28.1°C. However, as Fig.6 (percentage metamorphosis with time) shows, it is possible that the effect of temperature on larval growth continued after the temperature was changed. The effect of incubation temperature on *Macrobrachium rosenbergii* eggs and subsequent rate of larval development was demonstrated by Gomez-Diaz (1987) (section 2.3.3). He found that if the eggs were incubated at a lower temperature than that at which the larvae were reared, larval development was more rapid and more synchronous than if the eggs were incubated at the larval rearing temperature. Since egg incubation was at approximately 27°C in this trial, and initial larval rearing temperatures varied between 27.8- 28.6°C this might explain the more synchronous metamorphosis of the larvae in tank 3 (high bromide). This treatment also contained the highest stocking density, thus inadequate nutrition cannot be cited as the main reaon for the differences observed between this and the other treatments.

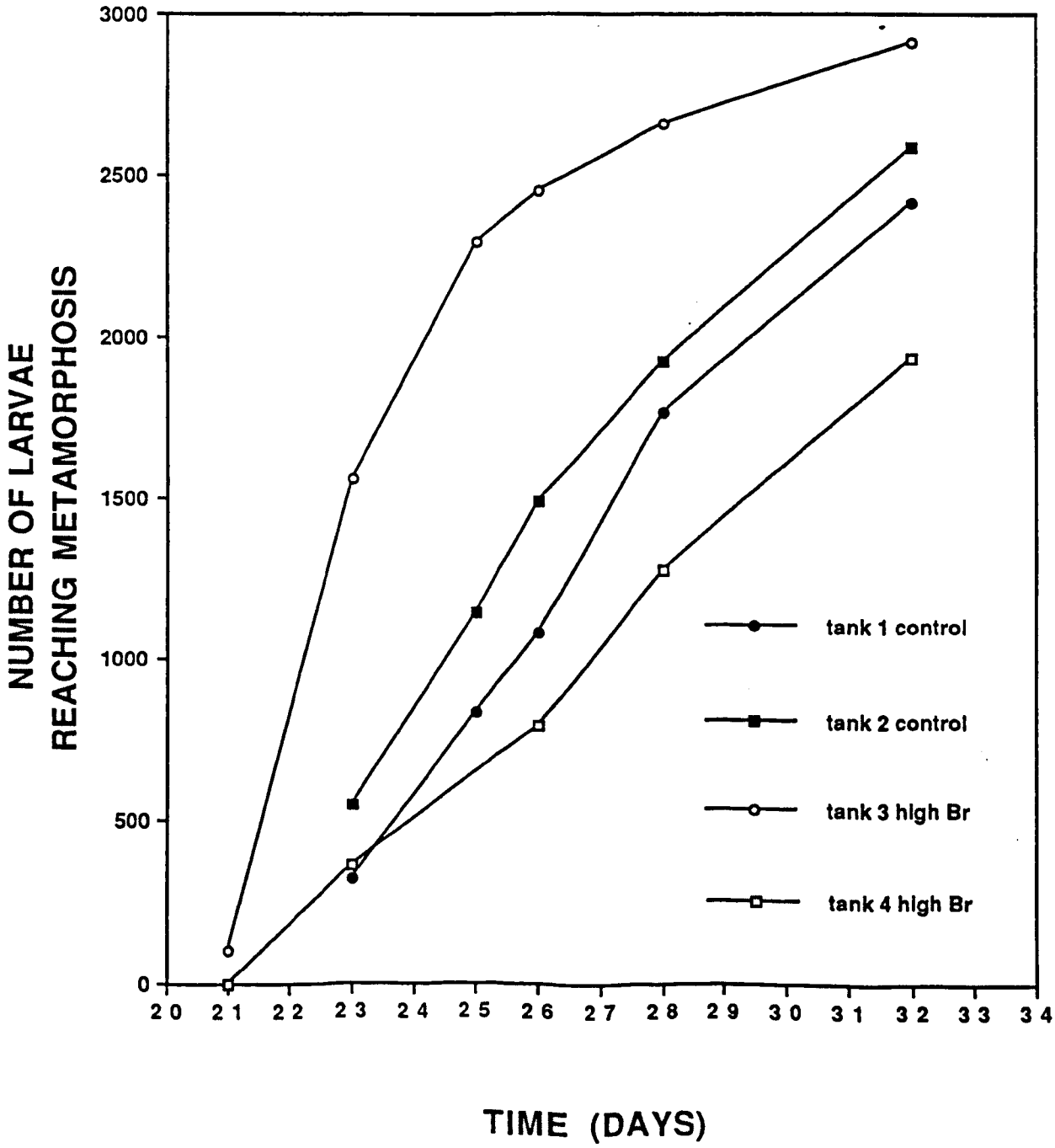
The conclusion that can be drawn from this rearing trial is that the addition of excess bromide to seawater does not have a profound effect on the growth or survival of *Macrobrachium rosenbergii* larvae. It would appear that the range of concentrations of bromide normally found in seawater are sufficient to ensure that bromide is not a limiting element to the developing *Macrobrachium rosenbergii* larvae. This is confirmed to some extent by the short term survival of larval *Macrobrachium rosenbergii* in artificial seawaters containing low bromide concentrations.

The observation that the larvae in the high bromide treatment seemed to have darker brown cuticles is intriguing. The darker brown colouration could be melanisation due to the stress of the high environmental concentration of bromide. This is unlikely since the growth and survival rates of the larvae in the two treatments were comparable. If the larvae were stressed it might be expected that the larvae in the high bromide treatment would not perform as well as those in the two controls. A second

(Fig.6) % metamorphosis of prawn larvae reared in high bromide and control treatments.



(Fig.7) Cumulative numbers of larvae reaching metamorphosis in control and high bromide treatments



possibility is that the bromide is affecting the formation of the cuticle. Since it is known that bromine is found in the scleroproteins of crustaceans and molluscs (sections 5.2,7.2), it is possible that the increased environmental concentration of bromide resulted in an elevated concentration of scleroprotein in the larval cuticle. These are known to have an amber colouration (section 7.2.1) and therefore be causing the darkening of the cuticle. A third possibility is that the high bromide concentration in the experimental treatment might have resulted in extra bromination of the scleroprotein of the cuticle, causing a darkening of its colour.

5.5 Conclusions

The requirement for bromide by the larvae of *Macrobrachium rosenbergii* seems to be linked to the moulting process. Whether this is because of an increased permeability of the larvae at the time of moulting causing excessive loss of the ion, or because the ion is directly required in the moulting process is unclear. The fact that the larvae are able to survive well with only trace additions of bromide to the rearing medium suggests a relatively efficient uptake mechanism, and thus the concept of unacceptable loss of bromide during moulting is probably erroneous. This leaves the possibility that bromide is direclty required as part of the moulting process. If this is the case it is almost certain that this role is concerned with the sclerotisation of the cuticle. Bromine could play a role in the modification of the physical properties of scleroproteins, or it might be influencing one of the enzyme systems involved in sclerotisation. If the larvae require bromide in their enviromental medium then this might be one reason why the larval stages of *Macrobrachium rosenbergii* are intolerant of freshwater.

CHAPTER 6 - Osmotic and ionic regulation.

6.1 Introduction

The invasion of freshwater from the brackish or marine environment presents several major problems to a crustacean. These are principally related to the chemical differences between brackishwater, seawater and freshwater, although there are also some physical problems. The chemical differences will influence osmotic and ionic control of body fluids, trace element availability, pH, oxygen carrying capacity of the water and reproductive strategy. Physical changes encountered include the unidirectional flow of water in rivers, causing displacement of planktonic forms, and the more variable temperature regime of terrestrial water bodies. The lack of marine algae (both micro and macro) could also cause a change in the nutritional composition of the diet.

In this section only the manner in which crustaceans tolerate freshwater and low or fluctuating salinity regimes will be considered. The other problems encountered in freshwater such as respiration, calcification, nutrition and reproduction are covered in other sections.

> *"Optimal cell function in active animals requires that the cellular environment has a well defined, relatively constant composition".*
>
> (Kirschner 1975)

The constancy of composition of the cellular environment is maintained by the extracellular fluids (ECF) which act as a buffer between the cells and the medium. The ECF are separated from the medium by epithelia in the crustaceans. However, these epithelia must be permeable to allow the exchange of gases, nutrients and waste products. Thus an aquatic crustacean can be considered as being 'leaky' to a range of solutes and to water. If an aquatic crustacean were to maintain the ionic composition of its ECF the same as the environment, then there would be no potential difference across the epithelial membrane. This is because the sum of the internal ionic charges would equal the sum of the external ionic charges. However, should there be a difference in total ionic charge

between the medium and ECF a potential difference would be generated (Fig.8) This potential difference would provide the energy for diffusive flow across the membrane. Thus, to maintain a steady state in the ECF the animal must generate an ionic counterflow to equal the rate of diffusion. This may be achieved by the use of an active transport mechanism, which in turn involves the expenditure of energy (Kirschner 1975). The greater the difference between the environment and the ECF, the greater the energy expenditure until a point is reached at which the organism is no longer able to counter the diffusive movement. This is due to a saturation effect on the transport mechanisms. At this point the maintainence of a steady internal state can be achieved only by a reduction in the permeability of the organism. A second problem that exists is that if there is an ionic gradient between the medium and ECF, then there will be an equal but opposite gradient for the diffusion of water. Thus an animal which is transporting salts in one direction must transport water in the other. In organisms that inhabit dilute media the ability to excrete a urine that is hypoosmotic to the blood is an important development in successfully controlling the ECF.

Crustaceans found in freshwater can be loosely grouped according to the manner in which they have adapted to freshwater, and the evolutionary time they have spent there. These groupings represent many independent invasions of freshwater (Mantel & Farmer 1983), however, the strategies employed are broadly the same. These strategies can be summarised as:

1) Reduction in blood osmolarity
2) Reduction in permeability
3) Ability to excrete hypoosmotic urine
4) Increase in ionic absorbtion

6.1.1 Osmotic regulation

The reduction in blood osmolarity is often employed by brackishwater species that osmoconform. In having a haemolymph osmotic pressure similar to the medium, the animal has only to contend with the osmotic pressure exerted on the tissues by the haemolymph. This is achieved by fluxes in ions and in the osmo-effecting free amino acids (Schoffeniels & Gilles 1970; Schoffeniels 1976). These are varied in response to changes in the osmotic pressure. The problem with osmoconformation is that

the tissues can only reduce their solute concentration to a limited extent before their function is impaired. Osmoconformers are not found in freshwater, which lead Mantel & Farmer (1983) to suppose that the lower salinity limit on osmoconformation lies in the organism's ability to vary its tissue solute concentration.

Table (XXIII) Osmotic pressure regulation in various *Macrobrachium* species.

Species		osmotic pressure (mosm.kg^{-1})							ref.
M. australiense	medium	0	260	500	700				a
	haemolymph	515	515	515	720				
M. rosenbergii	medium	0	500	800	965				b
	haemolymph	480	500	540	620				
M. rosenbergii	medium	10	160	230	380	490	680	840	c
	haemolymph	444	466	470	470	585	710	870	
M. ohlone	medium	10	120	265	440	520	650	800	c
	haemolymph	450	470	470	530	570	650	750	
M. carcinus	medium	10	200	400	600	800	1000		d
	haemolymph	460	465	475	500	710	925		
M.acanthurus	medium	10	200	400	-	800	1000		e
	haemolymph	420	445	525	-	705	875		
M. heterochirus	medium	10	200	400	-	800	1000		e
	haemolymph	440	460	500	-	750	850		
M. olfersii	medium	0	200	400	600	800	1000		f
	haemolymph	375	440	425	640	790	930		

a) Denne (1968), 2 days
b) Sandifer *et al.* (1975), 5 days, 28°C
c) Castille and Lawrence (1981), 5 days, 25°C
d) Moreira *et al* .(1988), 1 day, 20°C
e) Moreira *et al.* (1983), 1 day, 20°C
f) McNamara (1987), 1 day, 20°C

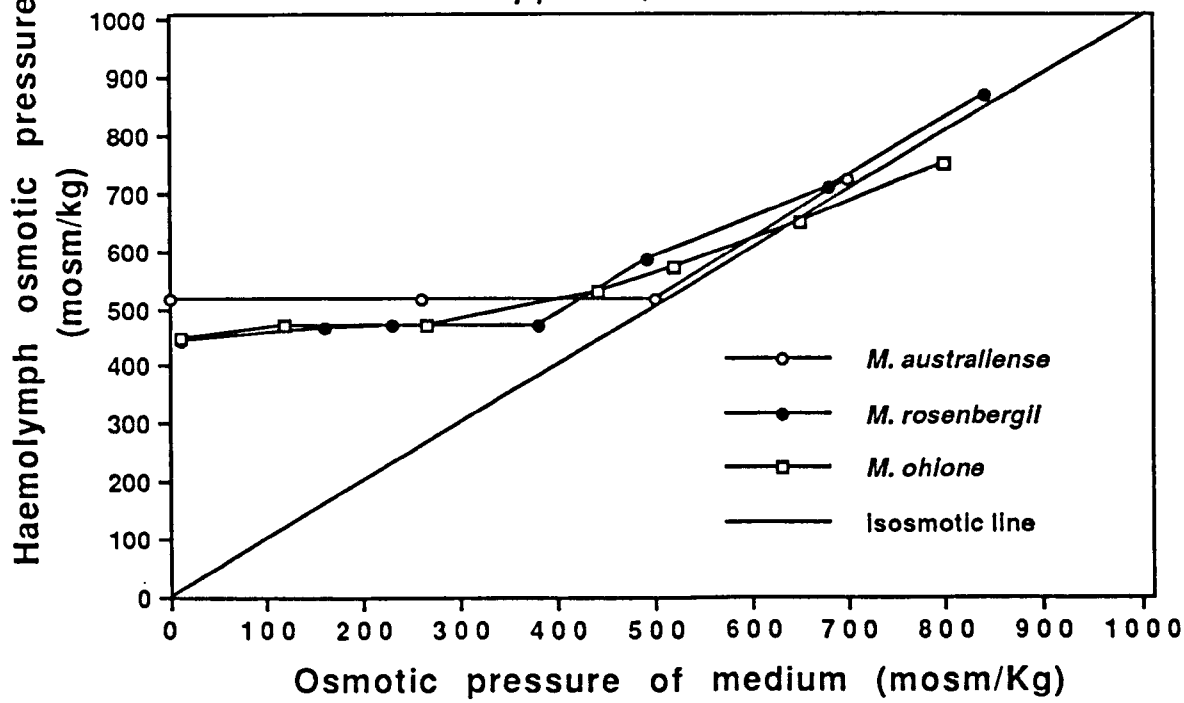
Freshwater species have reduced haemolymph osmotic pressures, although these are still maintained well above the medium. The osmotic pressure of the haemolymph

of crustaceans in freshwater can be used to divide them into two groupings. The first group contains the Branchiopoda and freshwater copepods, which maintain their haemolymph osmotic pressure at or below approximately 200 mosm.kg^{-1}. These species are thought to have had a long evolutionary history in freshwater resulting in them having a very low haemolymph osmotic pressure. The second group of freshwater crustaceans is composed of freshwater Malacostraca. These maintain their haemolymph osmotic pressure between 250-500 mosm.kg^{-1}when in freshwater. They can tolerate a seawater concentration of 50% seawater or more, and usually maintain their haemolymph hyperosmotic in all external salinities (Mantel & Farmer 1983). These features are also found in brackishwater crustaceans (Schoffeniels 1970) and thus appear to be 'conservative' among freshwater crustaceans (Lockwood *et al.* 1976).

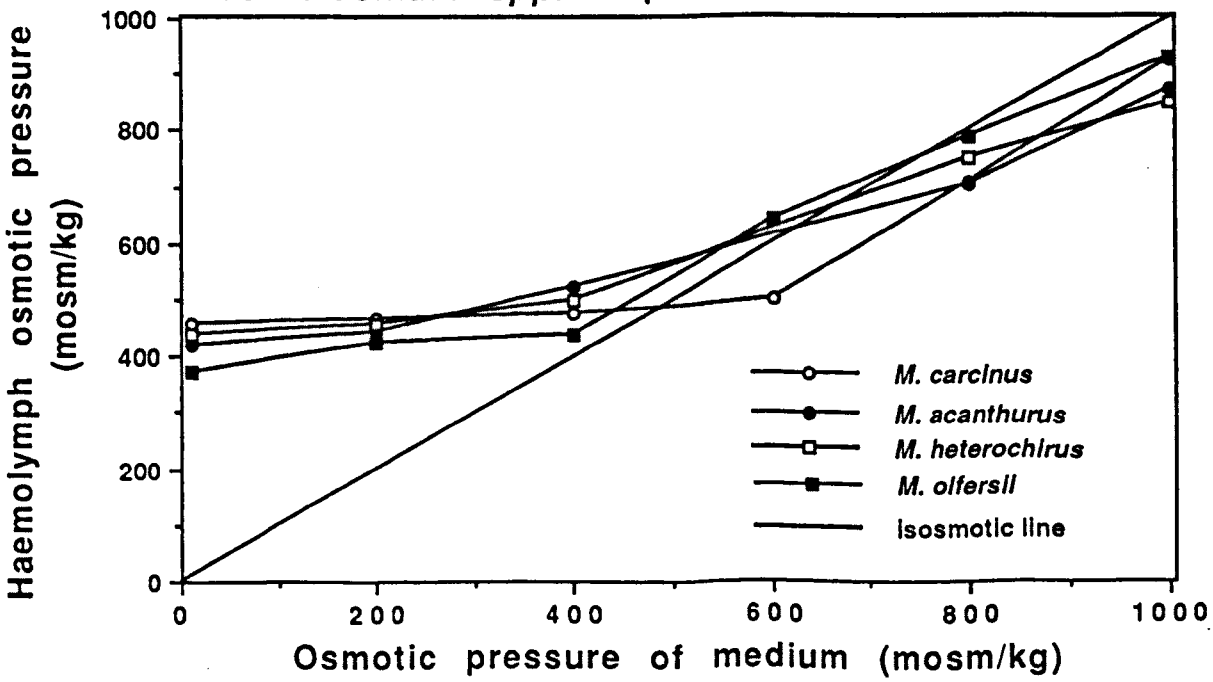
As can be seen from Table XXIII, and Figures 8 and 9, the regulation strategy of all of these species is the maintenance of approximately the same haemolymph osmotic pressure when exposed to increasing salinity, until the isosmotic point is reached. Above the isosmotic point, some species hyperregulate and others hyporegulate the blood osmotic pressure. This might be a function of acclimation time, since some of these results are for only 24 hours exposure. The values for *Macrobrachium rosenbergii* obtained by Sandifer *et al.* (1975) do not fit into this scheme. A possible reason for this is their use of postlarval prawns. These might have a wider range of osmotic regulation as they are newly metamorphosed from larvae. Harrison *et al.* (1981) reported that the haemolymph osmotic pressure of postlarvae decreased with time after metamorphosis (1-8 days). This contradicts the findings of Sandifer *et al.* (1975) who maintain that the osmotic pressure is the same in newly metamorphosed postlarvae and five month old prawns held at the same salinity. Clearly, there is some effect of acclimation here, as the quite strong hyporegulation found in the 5 month old prawns contradicts the findings of other authors who have studied the same species at shorter acclimation times (Castille &Lawrence 1981; Stern *et al.* 1987). Indeed, the osmotic regulation of salinity-shocked postlarvae reveals the adult type osmotic regulation in the same work by Sandifer *et al.* (1975).

There is evidence that some *Macrobrachium* species, including *Macrobrachium rosenbergii* can excrete a hypoosmotic urine when in dilute media (Denne 1968; Kamemoto & Tullis 1972; Stern *et al.* 1987). This implies the ability to reabsorb salts from the urine prior to excretion. Other palaemonid species seem able to excrete a marginally hypotonic urine (Parry 1957). The importance of excretion of a dilute urine

(Fig.8) Haemolymph osmotic concentration of various *Macrobrachium spp.* exposed to different salinities.



(Fig.9) Haemolymph osmotic concentrations of various *Macrobrachium spp.* exposed to different salinities.



in the ability to penetrate freshwater is unknown, as so few studies have been performed concerning this question (Mantel & Farmer 1983).

Possibly the best method of determining the degree to which the various *Macrobrachium* species have invaded freshwater is their reproductive strategy. Only in a few species do we see the ability to complete the lifecycle in freshwater and this is usually coupled with an abbreviation or loss of the larval stages (section 1.0), and a tendency to reduce the number of larvae. This method was first proposed by Sollaud (1923).

6.1.2 Ionic regulation

Table (XXIV) Haemolymph ionic composition of various Caridean prawns in full strength and dilute seawater.

Species	salinity (°/oo)	temp. (°C)	ionic concentration (mmol.dm^{-3}) Na	Cl	Ca	Mg	K	ref.
Pandalus hipsinotus	35	-	395	466	12.3	5.8	7.4	a
Palaemon serratus	35	-	395	428	12.5	12.6	7.4	b
Crangon crangon	35	10	410	421	11.5	9.1	-	c
Palaemon elegans	35	20	398	448	14.5	3.2	7.7	d
Palaemon longirostris	34	20	298	309	17.7	3.7	7.5	e
Ionic regulation in low salinity								
Crangon crangon	5	10	205	245	7.4	2.3	6.6	c
Palaemon elegans	5	20	179	205	7.6	0.5	3.8	d
Palaemon longirostris	3.5	20	279	271	13.4	2.1	6.3	e

references:
a) Mackay & Prosser (1970)
b) Parry (1954)
c) Hagerman (1973)
d) Ramirez de Isla Hernandez & Taylor (1985)
e) Campbell & Jones (1989)

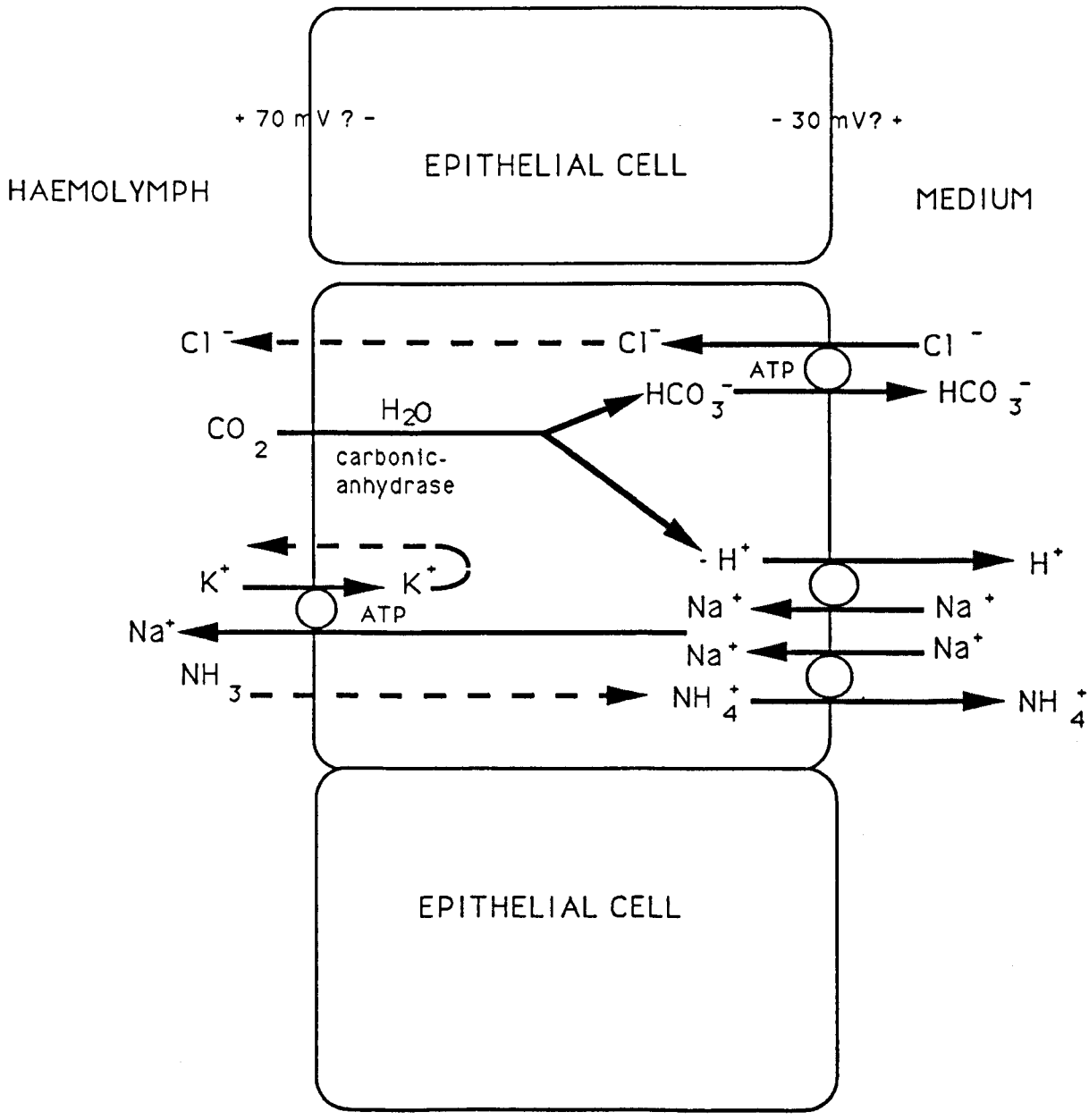
The ionic concentration of crustacean haemolymph is largely responsible for its osmotic pressure. Sodium and chloride constitute well over 90% of the ions in the haemolymph, thus their regulation forms the primary method by which a crustacean

regulates its osmotic pressure. The haemolymph ionic content, whilst often having a similar osmotic pressure, has a very different composition to that of seawater. The reasons for the hyperregulation of some ions and hyporegulation of others has been the subject of speculation in the many studies concerning ionic regulation in crustaceans. Table XXIV lists the haemolymph ionic concentrations of some palaemonid prawn species.

6.1.3 Sodium and chloride

These ions provide the bulk of the haemolymph osmotic pressure (HOP) although the presence of up to 10% protein in the haemolymph was also thought to contribute to the HOP (Mantel & Farmer 1983). However, in studying the colloid osmotic pressure of haemolymph proteins, Mangum & Johansen (1975) found that these contributed only 0.17 $mosm.kg^{-1}$ to the total osmotic pressure of the haemolymph. The mechanisms by which sodium and chloride are regulated in the body fluids of aquatic organisms are known to involve the expenditure of energy, and the participation of counter ions to maintain electroneutrality across the membranes (Fig.10). These counter ions are HCO_3^-, H^+, NH_4^+ and K^+ (Kirschner 1975). For a crustacean in a dilute medium, chloride ions are actively transported into the epithelial cell using HCO_3^- as an exchange ion. The HCO_3^- is derived from respiratory CO_2 via the action of carbonic anhydrase. Sodium is actively transported into the epithelial cell by Na/K ATPase using H^+ or NH_4^+ as the exchange ions. The H^+ is derived from the formation of HCO_3^- from respiratory CO_2, and NH_4^+ from haemolymph NH_3. The NH_3 and CO_2 are both excretory products.

Once the sodium and chloride are in the epithelial cell they then have to cross to the haemolymph. This is thought to be a passive process in the case of chloride, although it might be driven by an electrochemical gradient. Sodium movement into the haemolymph is active, potassium being the counter-ion. The potassium diffuses back into the haemolymph in a passive process. The transport mechanisms of sodium and chloride although linked, appear to be able to function independently of each other (Zanders 1981; Mantel & Farmer 1983). Stern *et al.* (1987) studied the effect of maintaining *Macrobrachium rosenbergii* in water of differing salinities and having exotic ionic



(Fig. 10) The supposed mechanisms for the active uptake of sodium and chloride in crustaceans. (from Kirschner 1975).

concentrations from different regions of Israel. The results of their analyses show that, provided the ionic ratios are not too dissimilar from those found in seawater, the prawn can maintain its blood at a constant composition. They found that all prawns maintained in a high magnesium and calcium medium died within 50 days. This period is greater than the usual length of the moult cycle in this species. Castille & Lawrence (1981) found *Macrobrachium rosenbergii* to be an efficient hyperregulator of its blood osmotic pressure (450 $mosm.kg^{-1}$), sodium (200 $mmol.dm^{-3}$) and chloride in freshwater and in salinities up to the isosmotic point. Thereafter, the prawn conforms with respect to the sodium concentration and osmotic pressure.

Ahearn (1978) reported that the epithelium of the intestine of *Macrobrachium rosenbergii* co-transported sodium and chloride together with calcium. The presence of calcium seems to be neccessary for the carrier protein to function correctly. The significance of this mechanism with respect to uptake via the gills is unknown, although it is possible that when in freshwater that the gut as well as the gills may be responsible for sodium and chloride uptake. This is not an unreasonable conclusion since the freshwater medium has a very low sodium and chloride concentration, whereas the food of the prawn will be comparatively rich in these ions. The significance of the diet upon the ability of freshwater prawns to osmoregulate correctly was demonstrated by Leinen (1982). When *Palaemonetes kadiakensis* were maintained in deionised water, and low sodium freshwater media, mortality was greater in unfed prawns. Prawns that were supplied with food maintained their haemolymph osmotic pressures and sodium concentrations.

The concentrations of sodium and chloride in the blood of brackishwater species usually differ. This is due to the requirement to maintain the blood ions in electrical balance as explained above. The chloride is usually present at a higher concentration than sodium to compensate for the reduced sulphate concentration. However, studies of some freshwater species indicates that they maintain the haemolymph sodium and chloride at very similar concentrations (Table XXV). This suggests that there is an anion present at high concentration to balance the calcium, potassium and magnesium concentrations (possibly sulphate or phosphate). Ahearn (1978) gives values for an intestinal incubation medium based on analyses of haemolymph and intestinal contents of *Macrobrachium rosenbergii*. In this medium, sulphate is included at a concentration of 25.1 $mmol.dm^{-3}$. It is not clear if this was the result of analysis or whether he obtained

the value by calculation of the difference between cations and anions. A concentration of 25.1 mmol.dm^{-3} for sulphate is very high indeed being equivalent to that found in 32^{o}/ooS seawater.

Table (XXV) **Concentrations of sodium and chloride in the haemolymph of some freshwater crustaceans. - adapted from Greenaway & Lawson (1982) and Mantel & Farmer (1983).**

Species	[Na^+]	[Cl^-]	Reference
	(mmol.dm^{-3})		
Orconectes limosus	245	240	Andrews (1967)
Orconectes rusticus	125	130	Sharma (1968)
Procambarus clarkii	185	185	Kamemoto *et al.* (1966)
Holthuisana transversa	270	265	Greenaway & Macmillan
Paranephrops planifrons	226	226	Wong & Freeman (1967a)
Paranephrops zealandicus	241	245	Wong & Freeman (1967a)
Pacifastacus leniusculus	212	209	Kerley & Pritchard (1967)
	199	170	Wheatly & McMahon (1982)
Cherax destructor	185	183	Greenaway & Lawson(1982)
Astacus astacus	191	199	Appelberg (1985)
Macrobrachium rosenbergii	175	165	Stern *et al.* (1987)

From Table XXV it can be seen that the concentrations of sodium and chloride in the haemolymph are similar but not identical. These slight differences could be attributed to analytical error. That these species have similar values is interesting as they are all (with the exception of *Macrobrachium rosenbergii*) members of the Astacoidea and Parastacoidea and all inhabit freshwater environments. It is possible that the close relationship between sodium and chloride concentrations is another adaptation to life in freshwater. The results obtained by Ahearn (1978) regarding the co-transport of sodium and chloride in *Macrobrachium rosenbergii* are especially interesting in the context of these similar sodium and chloride concentrations. Possibly the change from brackish to freshwater involves the change from predominantly gill mediated to predominantly gut mediated uptake of sodium and chloride. Kullama (1981) suggested that the gut played a regulatory role (although secondary) in *Macrobrachium rosenbergii.* It should be noted that there are other species within this group that do not have similar sodium and chloride concentrations and thus this feature may be coincidental, or not fundamental to the

invasion of freshwater.

6.1.4 Potassium

The requirement for potassium as the counter ion in the function of Na/K ATPase seems to be the reason for its presence in the haemolymph at the concentration found. Campbell & Jones (1989) comment on the fact that the majority of euryhaline crustaceans studied hyperregulate their blood potassium in salinities below 35 °/oo but that three species of palaemonid prawns do not. These species (*P. longirostris, P. serratus* and *P. elegans*) strongly hyporegulate potassium in salinities approaching full strength seawater. This observation holds true for all the caridean species in Table XXIV. Campbell & Jones (1989) conclude that the adaptive significance of this hyporegulation is unresolved. Mantel & Farmer (1983) suggest that the high potassium values obtained by some workers result from the use of whole haemolymph in their analyses, since the high potassium concentrations in the haemocytes would result in the overestimation of the serum potassium concentration (Potts & Parry 1964). Stern *et al.* (1987) found that *Macrobrachium rosenbergii* hyperregulates its blood potassium in salinities up to 24 °/oo. The potassium concentration in the haemolymph gradually increases with increasing salinity. Why this should happen is unclear, since the animal is conforming with respect to the sodium concentration. It cannot be explained by the increased activity of Na/K ATPase. Moreira *et al.* (1988) showed a similar response for *Macrobrachium carcinus* when exposed to varying salinity, with the prawn conforming completely to the medium at salinities above 22°/oo.

6.1.5 Calcium, magnesium and strontium

The divalent cations are regulated in a different manner to the monovalent cations, since they are often bound to organic or inorganic components of the haemolymph (Parry 1954; Robertson 1960; Mantel & Farmer 1983). This gives rise to the question of how much of the calcium and magnesium is present in ionic form in the haemolymph. Calcium is perhaps the best studied of the divalent cations since calcium salts are the principal mineral component of the crustacean cuticle (section 7.2.2). However, the majority of these studies have been concerned with fluctuations in the haemolymph

calcium concentration over the moult cycle. The involvement of calcium and magnesium in many enzymatic and respiratory processes suggests that the active ionic component of the haemolymph might be relatively constant, and that the major fluctuations observed over the moult cycle may be confined to the bound component. This is seen in *Austropotamobius pallipes* which has a total blood calcium range between 12.0-16.2 $mmol.dm^{-3}$ over the intermoult cycle, but the ionised component varies only between 6.0-7.4 $mmol.dm^{-3}$ (Greenaway 1972). Robertson (1953) reports that 10-20% of the blood calcium is complexed with protein in decapod Crustacea. Phosphate, sulphate and carbonate also bind calcium in the haemolymph. This has not been studied extensively. Greenaway (1976) found that the regulation of calcium in *Carcinus maenas* was not linked to the environmental calcium concentration but rather to the salinity of the medium. This is interesting since sodium and chloride regulation is linked to their concentration in the medium, and hence salinity (Zanders 1981). The concentrations of strontium and barium in the haemolymph have not been studied. Brannon & Rao (1979) comment that the calcium ATPases have a low specificity for calcium and hence may be responsible for the uptake of barium and strontium and their subsequent inclusion in the exoskeleton and soft tissues.

The effect of strontium on the calcification of the mollusc, *Aplysia californica* has been studied (Bidwell *et al.* 1986,1990). These authors found that strontium seems to play a role in the calcification process. Their conclusion was that it affected a biochemical pathway in the calcification process, and its absence prevented initial precipitation of calcium. Strontium did not appear to be essential for the correct formation of the crystal structure. Sub-optimal concentrations allowed normal calcification to occur.

The concentration of magnesium is kept well below the medium concentration in many crustaceans. This led Robertson (1953,1960) to suggest that it might be implicated in the nervous activity of crustaceans. His evidence for this was the comparatively high concentrations (80% of medium concentration) found in unresponsive crustaceans (*Hyas, Lithodes, Dromia*) and the lower levels (<50% of medium concentration) found in active crustaceans. Magnesium is thought to exert an anaesthetic effect on the neuromuscular junction (Katz 1936; Mantel & Farmer 1983). Thus the low haemolymph concentration of magnesium would aid the maintenance of a lower intracellular concentration. Guttman (1939) found that the alkaline earth metals (Ca,

Mg, Sr, Ba) had no effect on the injury potential of the non-medullated nerves of *Libinia canaliculata*. However, Treherne (1966) suggested that this cannot be the case, since subsequent work showed an effect of intracellular calcium, and elevated calcium levels in *Maia* were found to reduce the rate of potassium depolarisation. Guttman (1939) reported that Ca, Ba and strontium prevented the depressant effects of potassium and veratrine sulphate. The order of effectiveness in both cases was Ba>Sr>Ca. Lack of calcium caused spontaneous firing of multifibre preparations of lobster nerve (Gordon & Welsh 1948). Zanders (1980) found that exposure of *Carcinus maenas* to seawater containing an elevated magnesium concentration caused hyperexcitability, good muscle tone and good feeding. This contradicts the idea of the narcotic effect of magnesium, but is not commented upon.

Robertson (1953) suggested that the balance between calcium and magnesium is important. The evidence is that these two ions act antagonistically, magnesium inducing anaesthesia, whereas calcium induced excitibility. This was demonstrated by the addition of calcium to seawater containing *Carcinus maenas* (Bethe 1929 in Robertson 1953). Walters & Uglow (1981) studied the relationship between the relative heart activity and haemolymph magnesium concentrations in various crustaceans. They concluded that this technique was useful in qualifying the specific "activity" of the animals, since species with a high relative heart activity had the lowest haemolymph magnesium concentrations. Tentori & Lockwood (1990) found little evidence to support the theory of magnesium affecting activity in the oceanic crustaceans they studied. The species ranged between passive planktonic to those which underwent substantial diurnal migrations.

Stern *et al.* (1987) found that *Macrobrachium rosenbergii* grown in an exotic saline media died in a treatment containing high magnesium. The magnesium concentration was twice that of seawater of the same salinity. They do not comment upon the fact that the calcium concentration in the medium was nearly five times that of seawater at the same salinity! The mortality of the prawns may have resulted from ionic imbalance rather than actual lethal concentrations of magnesium.

Winkler (1986) demonstrated the effect of altered calcium to magnesium ratios on Na/K ATPase in *Carcinus maenas* gill preparations (*in vitro*). At ratios corresponding to those found in seawater the ATPase functioned maximally. At ratios with

low magnesium and high calcium there was almost complete inhibition of enzyme activity.

Magnesium has also been implicated in the maintenance of structure of haemocyanin subunits (review by Mangum 1983). It seems that the polymerisation of crustacean haemocyanin is stabilised by the presence of magnesium and calcium. The two ions appear to be interchangeable, and which ion has the most significant effect depends upon their respective haemolymph concentrations. In some Thallassinid species which have a low haemolymph calcium concentration (10 $mmol.dm^{-3}$) but a high magnesium concentration (50 $mmol.dm^{-3}$), the magnesium exerts the effect, as calcium alone would favour the dissociation of the haemocyanin eicositetramers, dodecamers and hexamers into monomers. The concentration of calcium and magnesium required for stabilisation of haemocyanin in other species seems to be well below the lower physiological limit, thus it would seem that the concentration in the blood of these ions is maintained for other reasons.

6.1.6 Copper

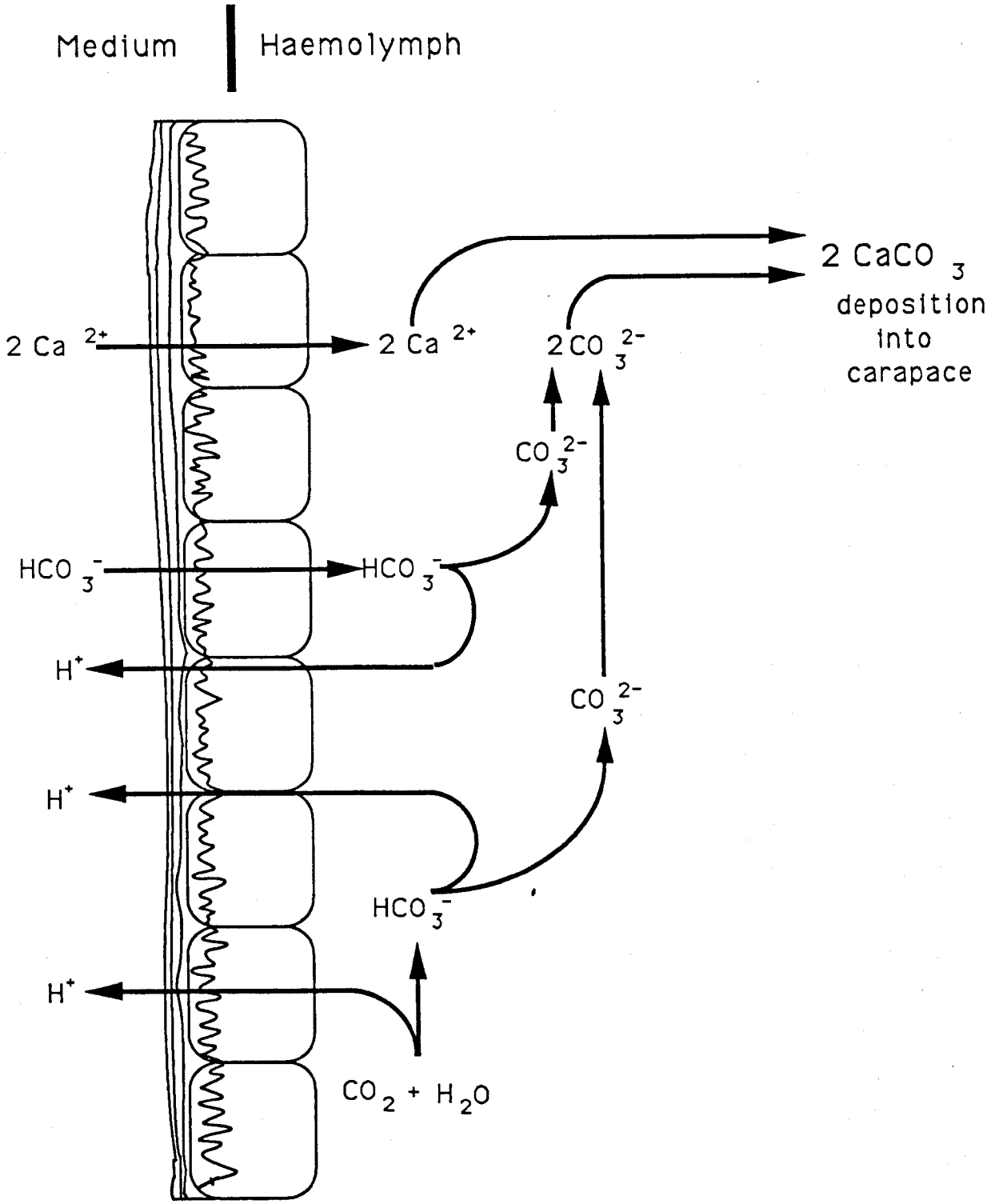
In crustaceans the presence of copper is confined mainly to the haemolymph and the hepatopancreas (Djangmah & Grove 1970). The haemolymph copper content is due to the copper containing respiratory pigment haemocyanin. Haemocyanin forms the main, but not the only protein component of the haemolymph; at intermoult this is approximately 80-95% of the total protein (Kerkut *et al.* 1961; Schoffeniels 1976; Smith & Dall 1982; Ghidalia 1985). Thus haemocyanin represents the bulk of the osmoeffecting organic material of the haemolymph. Any fluctuations in the concentration of haemolymph protein should be reflected as a change in the haemocyanin concentration.

When a euryhaline crustacean is subjected to hyperosmotic stress there is an increase in the intracellular free amino acids, and corresponding decrease in haemolymph protein, as cells compensate for an increased haemolymph osmotic pressure (Schoffeniels 1976). Thus in increasing salinity there is a tendency to reduce haemolymph haemocyanin.

6.1.7 Effect of moult cycle

The crustacean intermoult cycle is a dynamic process that periodically involves the shedding of the exuvia, this causes changes in permeability and hence variations in ionic concentration and osmotic pressure. Changes in the ionic composition of the haemolymph throughout the moult cycle have been studied, although this work has been principally concerned with osmotic, calcium and magnesium regulation. The haemolymph of *Crangon crangon* becomes concentrated from postmoult until premoult (Hagerman 1973). This is seen as a gradual increase in the concentration of sodium in the haemolymph. The concentration of protein and organic substances also increases towards premoult (Mantel & Farmer 1983). At the moult, the permeability of the crustacean increases allowing the osmotic influx of water (Passano 1960). This causes the swelling of the animal and subsequent splitting of the exuvia, allowing removal. After moulting, the cuticle is hardened by the processes of sclerotisation and calcification (section 7.2). The deposition of calcium into the cuticle continues for some time after a crustacean has moulted (Greenaway 1974; Vigh & Dendinger 1982). Cameron (1985) proposed that the transport of calcium into a crustacean was electrically balanced by the excretion of hydrogen ions. The import of bicarbonate and use of respiratory carbon dioxide provided the carbonate for calcium carbonate deposition during calcification (Fig. 11).

Calcium is stored in the hepatopancreas of *Macrobrachium rosenbergii* and haemolymph calcium concentrations are also elevated prior to moulting (Fieber & Lutz 1982). In *Crangon crangon* the haemolymph concentration of calcium decreases towards the time of moulting, and this is attributed to storage in the midgut gland. The haemolymph magnesium concentration is increased at the moult and is also high in early premoult. This increase is presumed to be due to an increased diffusion into the animal due to its increased permeability and the high environmental concentration in seawater, this is corrected as soon as the postmoult stage is reached. Urinary excretion of magnesium increases during premoult and postmoult in *Crangon crangon* as the prawn attempts to reduce the influx from seawater (Hagerman 1973,1980). However, Fieber & Lutz (1985) demonstrated an increase in the concentration of both calcium and magnesium prior to moulting in *Macrobrachium rosenbergii* when in freshwater. In this environment the external concentration of magnesium would favour ion loss from the prawn by diffusion. This is in contrast to the hypothesis of Hagerman (1973), who believed that the high magnesium concentration was due to the inability of *Crangon crangon* to excrete the



(Fig. 11) A diagrammatic scheme of electrically and acid-base balanced transport of Ca^{2+}, HCO_3^- and H^+ in the postmoult crab. (Redrawn from Cameron 1985).

magnesium entering from the environment. In *Macrobrachium rosenbergii* the high concentration of magnesium during moulting would seem to be actively maintained. Fieber & Lutz (1985) attributed this to two possible reasons: 1) the higher level of magnesium increases the affinity of haemocyanin for oxygen, or 2) the enzymes that are involved in moulting require higher levels of magnesium to function effectively due to the marine origins of the species.

Kullama (1981) demonstrated the ionic/osmoregulatory role played by the midgut in *Macrobrachium rosenbergii* during the intermoult cycle. It seems the sodium and sodium linked water uptake occurs in the midgut, although this may not be the primary mechanism. The permeability of the gut changes over the intermoult cycle causing a 5-10% increase in osmolarity and concomitant increase in sodium and chloride concentration in premoult animals. A similar sized decrease in all these parameters occurs immediately postmoult.

Copper concentrations vary over the course of the moult cycle and this is generally assumed to reflect the haemolymph concentration of copper. Zuckerkandl (1960) found that immediately following the moult haemocyanin concentration is very low in *Maia squinado*. This low level continues into early intermoult and then increases to a maximum in late intermoult, where it remains until the next moult. There is a drastic fall just after moulting and the cycle is then repeated. The fall in haemolymph haemocyanin concentration is correlated with a rise in hepatopancreas copper concentration, and it is assumed that, as haemocyanin is broken down during the moult, storage in the hepatopancreas is occurring. Not all of the copper is stored but some is excreted (Kerkut *et al.* 1961). Djangmah & Grove (1970) found a similar pattern of copper regulation in *Crangon crangon*. There was a difference with respect to the stability of the copper concentration over the intermoult and premoult period, unlike *Maia* the copper concentration rose to a premoult maximum in stage D_2/D_3, and then decreased to a minimum at ecdysis. The range of copper concentrations was between 0.865 $mmol.dm^{-3}$ and 2.12 $mmol.dm^{-3}$.

6.2 Adult *Macrobrachium rosenbergii* osmotic and ionic regulation in different salinities.

In this study it was hoped to elucidate more fully the degree to which *Macrobrachium rosenbergii* has adaptated to life in freshwater. In particular the manner in which the elements strontium and bromine are regulated. These elements are found in seawater and are readily available to marine organisms due to their relatively high concentration in seawater. This is not so for animals inhabiting the freshwater environment, and it would be of interest to see whether the animal attempts to retain the ions, or allows the environmental concentration to determine the level in its haemolymph. This is of particular interest since neither of these ions have been proven to be essential, and thus their regulation might be taken as an indication of essentiality.

6.2.1 Materials and methods

Intermoult prawns were exposed to a range of salinities between freshwater and 28°/ooS. Full strength seawater was diluted with deionised water to give the desired salinity. The prawns were held in individual plastic pipes (10cm diameter). The pipes had a coarse mesh at either end to allow a flow of water through them. The pipes were placed in a $70dm^{-3}$ conical tank filled with seawater at the desired salinity. The seawater was recirculated through the holding tank using an 'Eheim powerfilter'. Salinity was measured using a hand held refractometer accurate to ±1°/ooS. Aeration was provided via airstones and the temperature maintained at 28 ± 1°C using aquarium heaters. The 'Eheim' recirculating biological filter contained a mixture of crushed cockle shell and gravel (ratio by volume ≈1:2). The biological filter was flushed with freshwater between each treatment and allowed to equilibrate to each new salinity before the prawns were introduced. Prawns were fed lightly for two days after exposure, and then starved until after sampling.

The prawns were maintained in each test salinity for seven days. The prawns were introduced straight from freshwater into salinities up to 18 °/oo. With higher salinities the prawns were acclimated for 24 hours at 18 °/oo before being introduced to the higher test salinity. Eight adult prawns (≈20-40g) were used for each

exposure, and the same prawns were not used more than once within the same moult cycle. The reason for not resampling prawns was to avoid the possibility of depleting the animal of of the bromine/bromide and strontium. The manner by which, and how rapidly, these ions are obtained is unknown, therefore repeated sampling might have given erroneous results. Another reason for not resampling the prawns was the fear of killing the animals, which were in short supply. In order to ensure that sufficient haemolymph could be obtained for the analyses, prawns weighing ≈20g or more were required. It took approximately four to five months to rear animals of this size from postlarvae. Since the prawns could not be sampled more than once per moult cycle, and all the prawns used had to be in intermoult, there were few suitable animals available at any one time. This was due to their lack of synchrony in moulting, and had the effect of allowing only one salinity exposure per month.

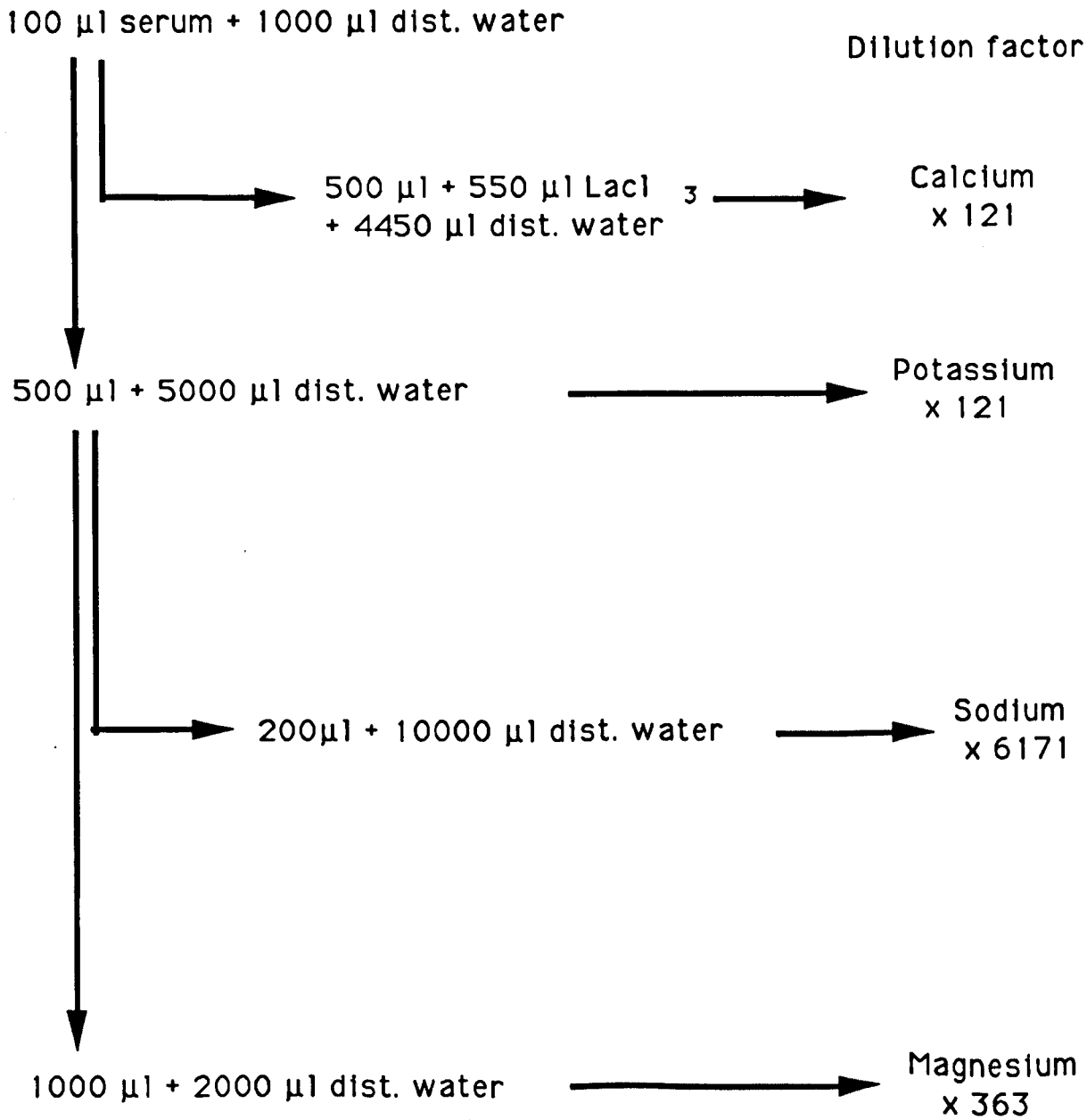
Prawns were sampled and the haemolymph was treated as detailed in section 3.3. Three separate series of salinity exposures were performed to provide the haemolymph for the following analyses.

In all experiments samples of the exposure medium were taken at the time of sampling, and kept frozen until required for analysis. Haemolymph and seawater samples were always analysed concurrently.

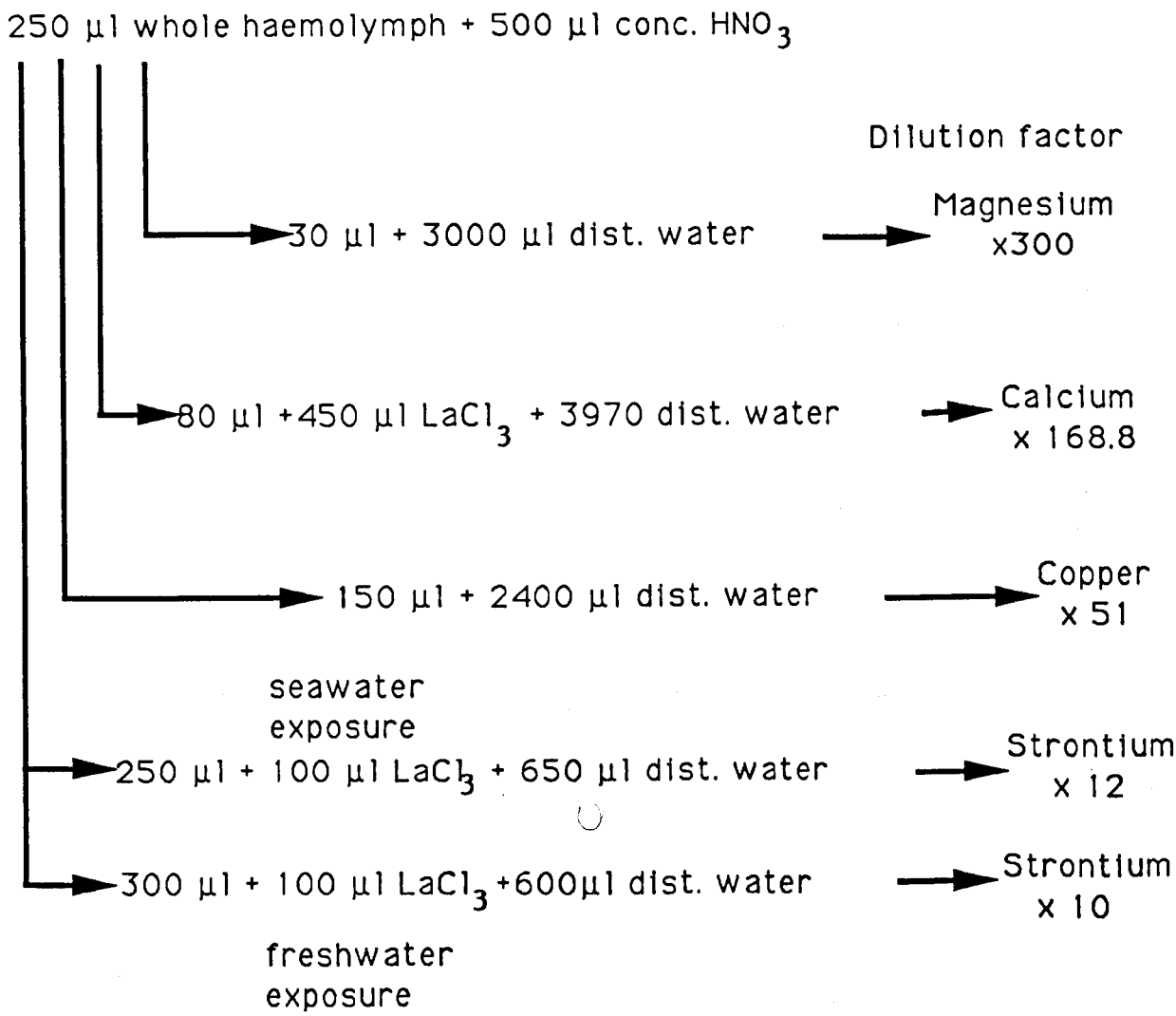
Experiment 1) Prawns were exposed to freshwater and five different salinities. This provided haemolymph for serum Na, K, Ca, Mg, Cl, and osmotic pressure analysis. The serum concentrations of these ions were intended to provide information as to the manner in which *Macrobrachium rosenbergii* maintains its haemolymph ionic environment between freshwater and various salinities. Serum was diluted for ionic analysis using AAS according to the protocol in Fig.12, and seawater was diluted according to the protocol in Fig. 13.

Experiment 2) Prawns were exposed to freshwater and five different salinities as in experiment 1. In this experiment whole haemolymph was analysed for the concentrations of Ca, Mg, Sr, and Cu. The reason for using whole haemolymph was to try to determine the degree to which these ions were bound in the haemolymph such that they might not be detected in the serum analyses. In the case of copper the majority is known to be bound as haemocyanin and fluctuations in its concentration could indicate a change in the

Fig. 12 Dilution protocol for ionic analysis of serum of prawns exposed to varying salinities.



(Fig. 13) Digestion and dilution protocol for whole blood analysis of prawns maintained in varying salinities.



(Fig. 14) Dilution protocol for the ionic analysis of seawater.

	Dilution factor
1) 10 µl seawater + 10000 µl dist. water →	magnesium x 1001
2) 1000 µl + 5000 µl dist. water →	sodium x 6006
3) 100 µl seawater + 500 µl $LaCl_3$ + 4400 µl dist. water →	calcium x 50
4) 100 µl seawater + 10000 µl dist. water →	potassium x 101
5) 100µl seawater + 1000 µl dist. water →	chloride x 11

(Fig. 15) Dilution protocol for the ionic analysis of seawater. (Acid digested samples).

Dilution factor

1) 10 µl seawater + 9300 µl dist. water + 700 µl HNO_3 → magnesium x 1001

2) 100 µl seawater + 500 µl $LaCl_3$ + 4340 µl dist. water + 60µl HNO_3 → calcium x 50

3) 1000 µl seawater + 400 µl $LaCl_3$ + 660µl HNO_3 + 1940 µl dist. water → strontium x 4

concentration of haemocyanin when the prawns were exposed to seawater of differing salinity. The whole haemolymph samples were digested in nitric acid to solubilize any bound metal. Sample dilution for AAS was performed according to the protocol in Fig. 14, and seawater dilution according to Fig. 15.

Exposure 3) Prawns were exposed to freshwater and five different salinities. Whole haemolymph was used for this experiment as it was unknown whether the bromine/bromide was present ionically or in a bound form. Samples of haemolymph (250µl) and seawater (250µl) were dried for the activation analysis of the bromine/bromide content (section 3.3.7).

6.2.2 Results and discussion

All graphs were plotted as means ± the standard error of the mean. Where stated, two sample t-tests were performed using 'Statgraphics', a computer statistical package.

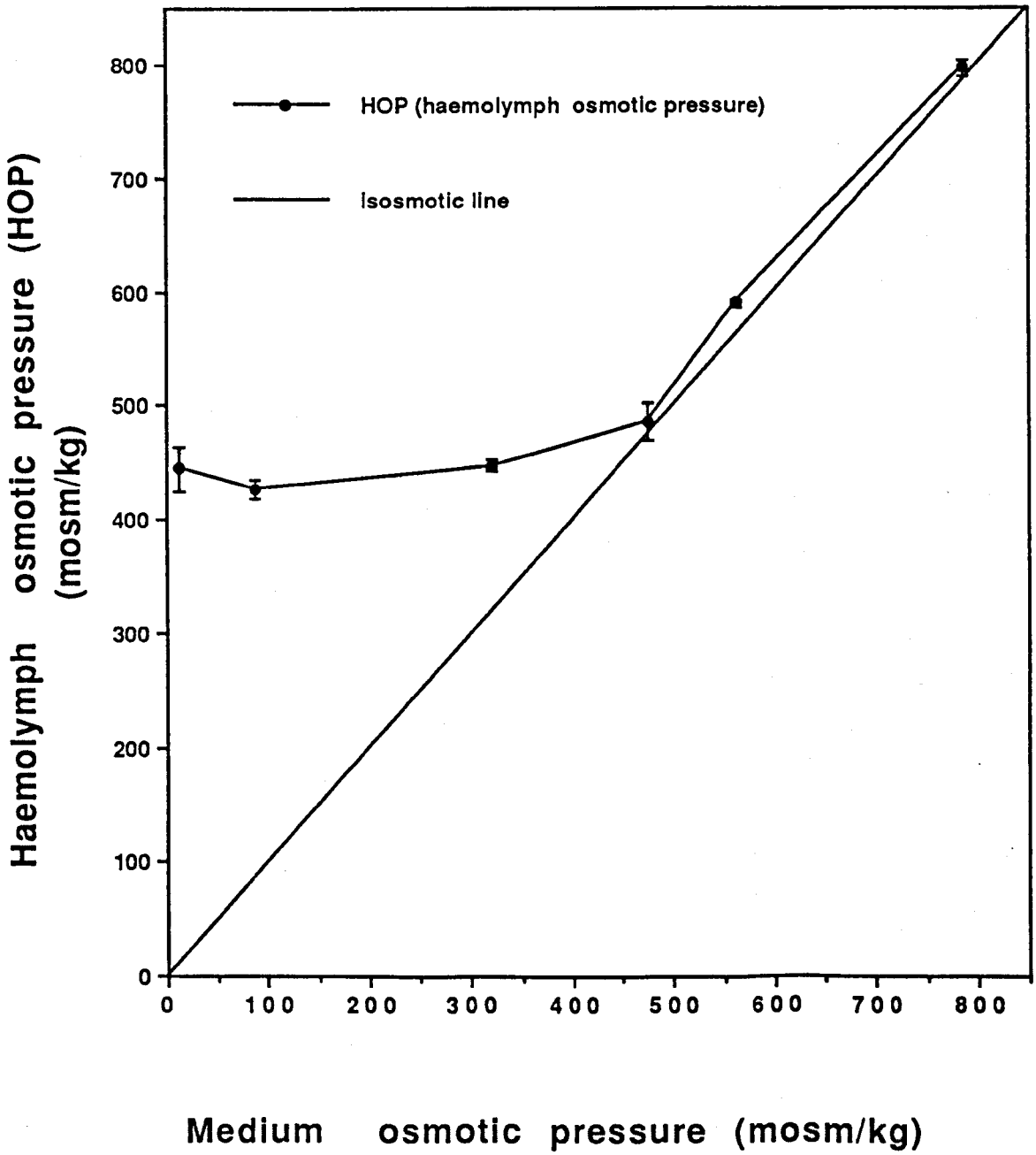
6.2.2.1 Osmotic pressure (Fig. 16)

Macrobrachium rosenbergii showed very strong hyperregulation of its haemolymph osmotic pressure (HOP) over a salinity range of 0-15^{o}/oo. The HOP over this range has a mean value of 451.8 mosm.kg^{-1}. A two sample t-test showed no significant difference ($p<0.05$) between the HOP values determined over this range. As the osmotic pressure of the medium exceeded its isosmotic point, *Macrobrachium rosenbergii* hyperconformed its HOP very slightly above that of the medium.

This pattern of osmoregulation in varying salinity is typical of most of the *Macrobrachium* species studied to date (section 6.1.1). There does appear to be some interspecific difference in the capacity to maintain a constant HOP in increasing salinity. However, these differences might be attributable to the different acclimation times in the various experiments. In this experiment the prawns were allowed one week to acclimate to the test salinity, in many of the others acclimation times were less.

Castille & Lawrence (1981) obtained similar results in their study of the

(Fig.16) HOP of adult prawns held for seven days at different salinities.



HOP in varying salinity of *Macrobrachium rosenbergii*. The hyperconformation of the HOP in salinities above the isosmotic point is suggested to be the trait of a truly freshwater crustacean (Mantel & Farmer 1983).

The HOP of *Macrobrachium rosenbergii* in freshwater (≈450 mosm.kg^{-1}) is higher than the HOP's of the 'true' freshwater crustaceans, that have had a long evolutionary history in freshwater (Mantel & Farmer 1983). *Macrobrachium rosenbergii* exhibits the osmoregulatory traits of the euryhaline Malacostracans. However, its ability to withstand frshwater as a natural habitat separates this species from its palaemonid relatives. None of the species listed in Table XXIV can withstand completely freshwater. Thus *Macrobrachium rosenbergii* has developed mechanisms for withstanding the osmoregulatory and ionoregulatory stresses of freshwater. This is not so in the larval stages of this species, and these abilities appear to become functional at metamorphosis.

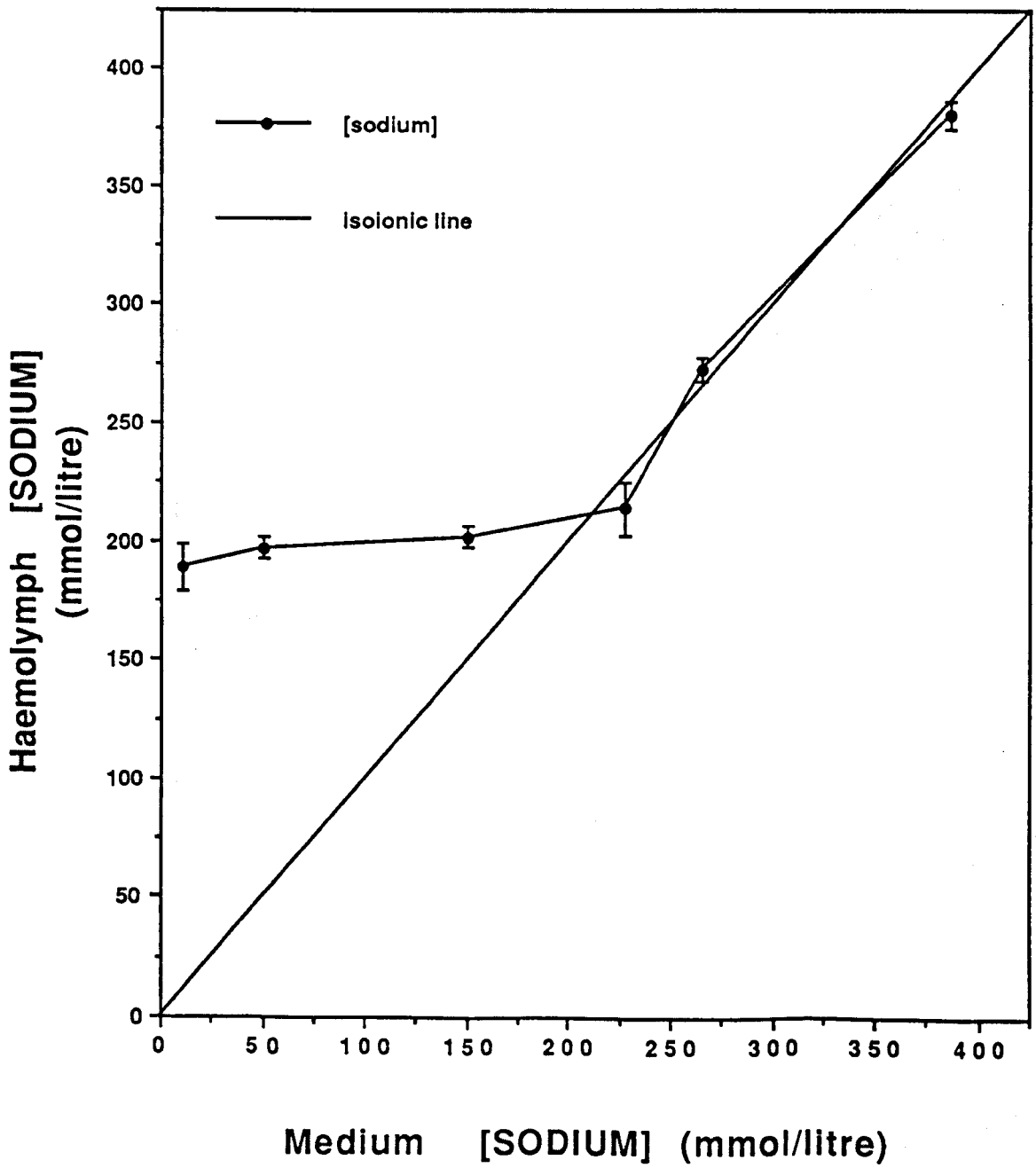
6.2.2.2 Sodium and chloride (Figs. 17,18,19)

The haemolymph sodium concentration was hyperregulated in salinities below the isoionic point (0-15^{o}/oo) at a concentration of approximately 200 mmol.dm^{-3} (Fig.17). Over this range there was no significant difference between any of the haemolymph sodium concentrations. In freshwater the sodium concentration of the haemolymph was slightly reduced to a value of 188 mmol.dm^{-3}, although this difference was not found to be significant (two sample t-test, $p<0.05$) In salinities above the isoionic point the haemolymph sodium concentration hyperconformed to that of the medium.

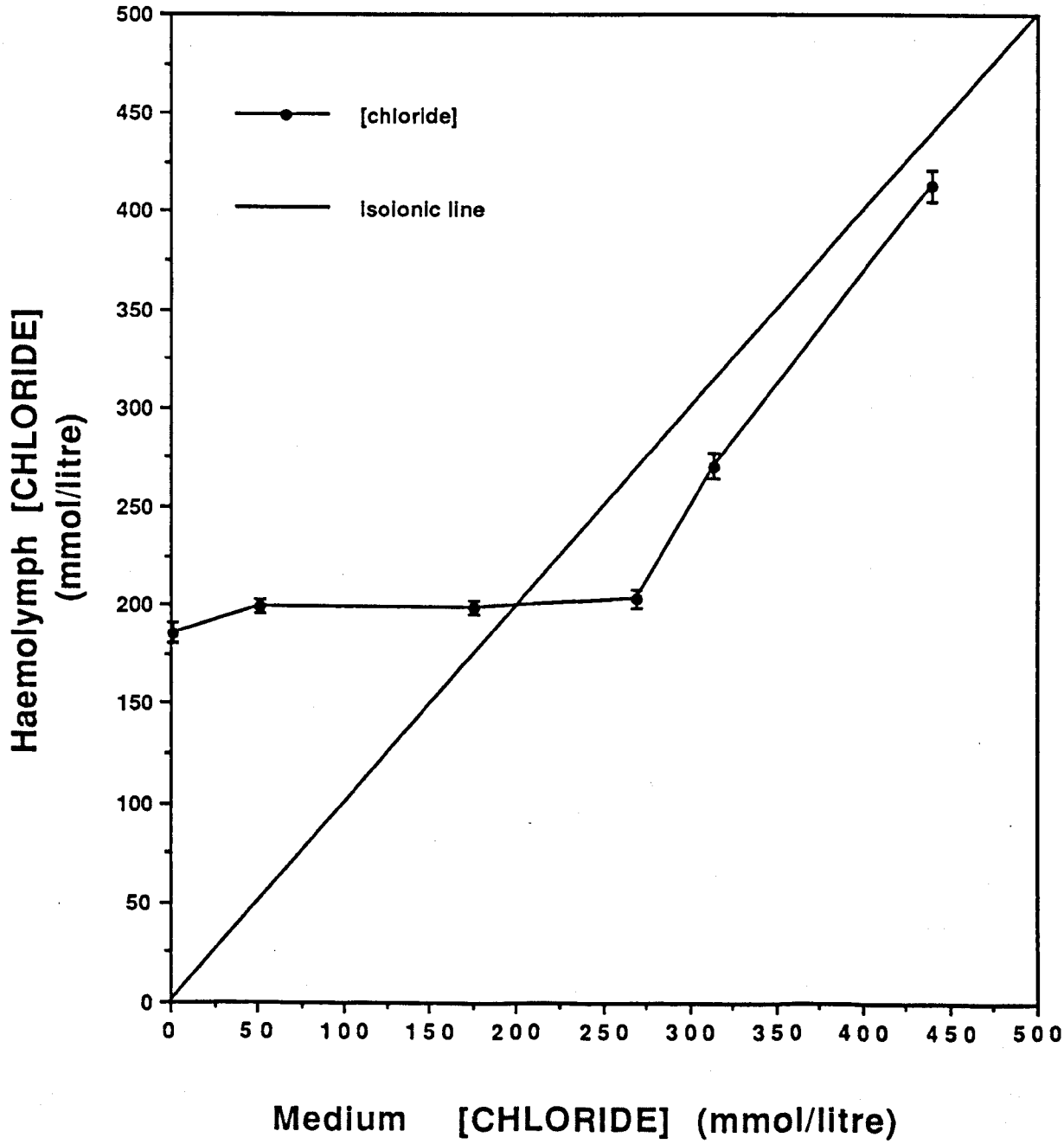
The haemolymph chloride concentration was hyperregulated up to and beyond the isoionic point at a concentration of approximately 200 mmol.dm^{-3} (Fig.18). The haemolymph chloride concentration was slightly reduced in freshwater to a level of 186 mmol.dm^{-3}. Above a medium concentration of approximately 270 mmol.dm^{-3} the chloride concentration increased, but remained hypoionic to the medium.

If the haemolymph sodium and chloride concentrations are plotted against salinity the two are almost superimposed (Fig. 19). Apart from the highest salinity exposure there is no significant difference between the haemolymph sodium and chloride concentrations. At the highest salinity tested (26^{o}/oo) the chloride concentration is

(Fig.17) The haemolymph [Na] of adult prawns held at different salinities.



(Fig.18) Haemolymph [Cl] of adult prawns held at different salinities.



slightly reduced from that of the sodium.

This close relationship between sodium and chloride, even in freshwater, implies that the regulation of these two ions may be linked. If this is so then there must be another mechanism responsible for the maintenance of haemolymph osmotic pressure. This is because the contribution made by sodium chloride to the total haemolymph osmotic pressure does not account for the observed osmotic pressure at any of the salinities studied. Table XXVI shows the contribution to total haemolymph osmotic pressure made by the combination of sodium and chloride in the haemolymph.

Table (XXIVI) The contribution of sodium chloride to the haemolymph osmotic pressure in adult *Macrobrachium rosenbergii*.

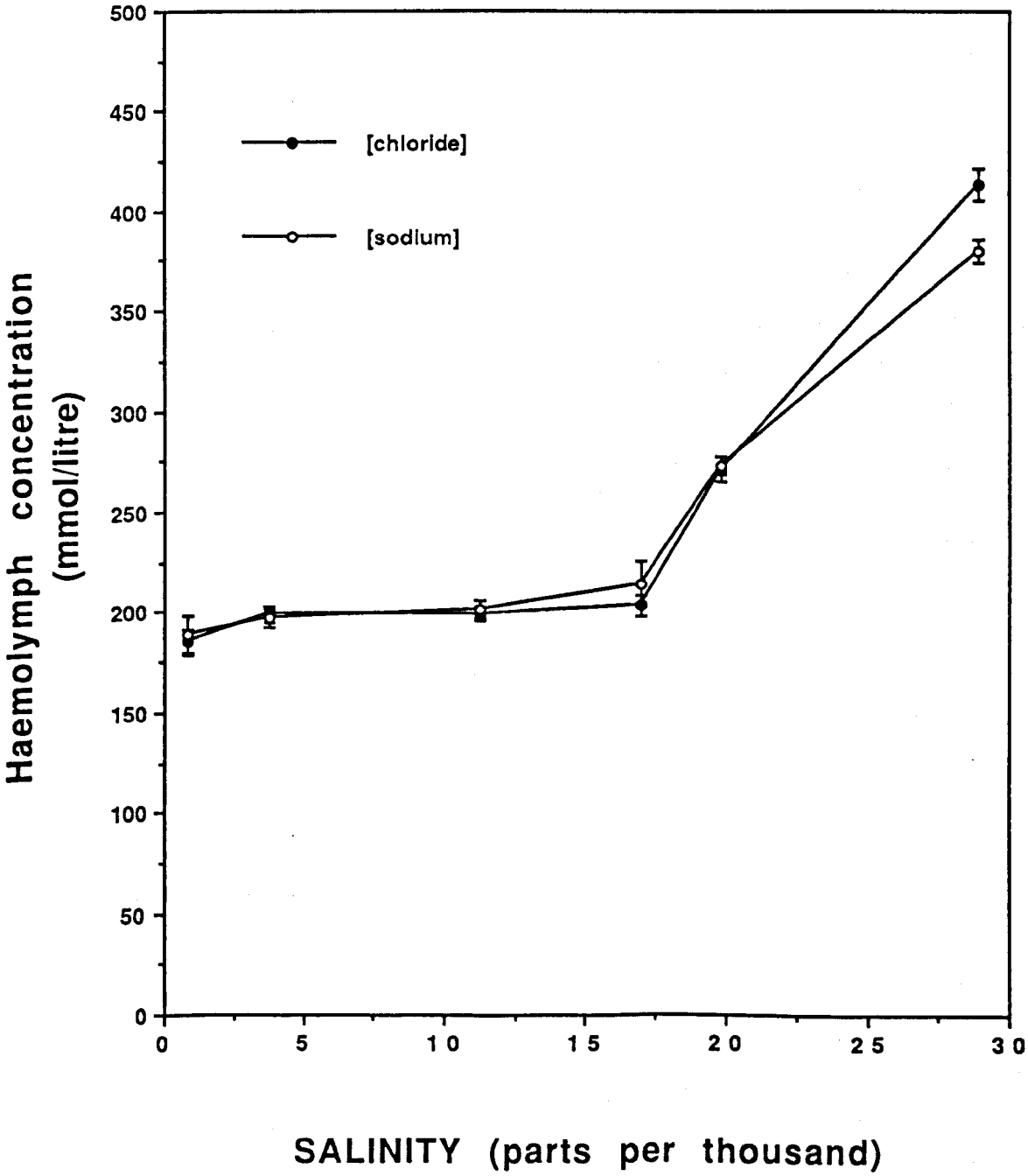
	SALINITY (°/oo)					
	29	20	17	11	4	0
sodium chloride ($mmol.dm^{-3}$) in prawn	380.4	272.9	214.2	201.9	197.0	188.4
osmotic pressure ($mosm.kg^{-1}$) in prawn	797.8	590.4	486.6	448.4	427.3	444.8
osmotic pressure ($mosm.kg^{-1}$) provided by NaCl *	699.2	503.3	395.6	373.2	364.8	349.9
difference ($mosm.kg^{-1}$)	98.6	87.1	91.0	75.2	62.5	94.9

* taken from Wolf *et al* .(1980)

The difference in the osmotic pressure between that provided by sodium chloride and the measured value, will be provided by other ions and organic compounds in the haemolymph.

The apparent linkage between the sodium and chloride concentrations in the haemolymph of *Macrobrachium rosenbergii* suggests that there might be a primary controlling factor that synchronizes the two uptake systems. Zanders (1981) suggested that ionic regulation in *Carcinus maenas* involved the linkage of a number of the ionic uptake mechanisms. He argued that this enabled the ionic regulation of the animal to be

(Fig.19) Relationship between the haemolymph [Na] and [Cl] in adult prawns held at different salinities.(27 °C).



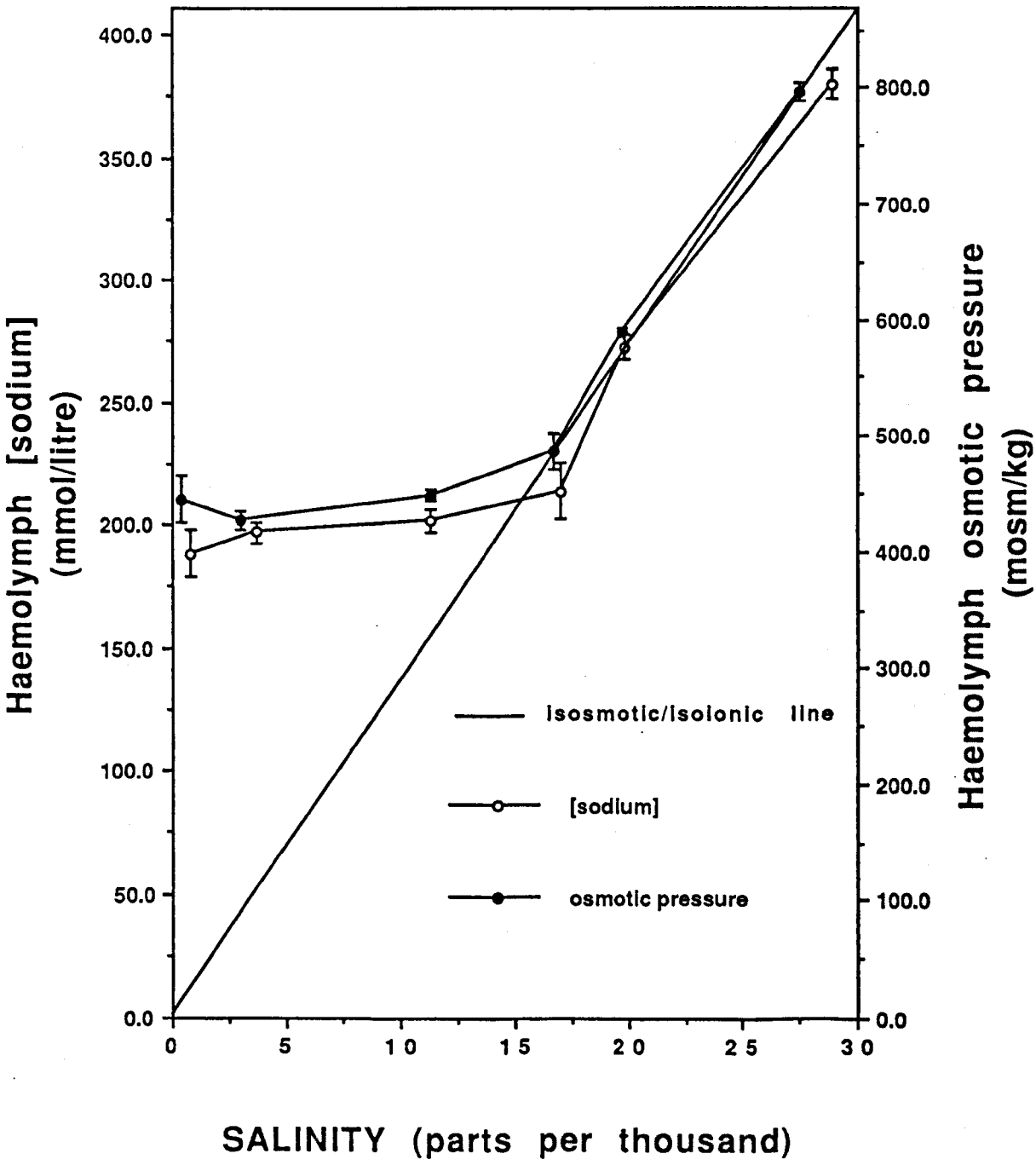
controlled by one factor. This factor was able to control the ionic regulation in this way due to the constant ratio of ions in seawater and brackishwater. Such a system could not possibly be effective in freshwater where no such ratio between the ions exists. However, Zanders (1981) also found that the sodium and chloride uptake mechanisms could operate independendently of each other. He suggested that the mechanism by which these ions were maintained in their correct relative proportions involved the inhibition of uptake of an ion that was in excess until the correct ratio was re-established. Once the correct ratio had been achieved, the overall controlling mechanism would then maintain the ions at the concentrations required to maintain the haemolymph osmotic pressure at the desired concentration. The suppression of uptake was supposed to be caused by the change in trans-epithelial potential difference caused by the imbalance of sodium and chloride ions.

The 1:1 ratio between the sodium and chloride concentrations that is maintained in dilute seawater and freshwater cannot be a function of the environmental ionic ratio. This is shown by the maintainance of the 1:1 ratio in freshwater where the medium sodium and chloride concentrations are not in any fixed ratio. Similarly, the regulation of the uptake mechanisms for sodium and chloride by changes in trans-epithelial potential difference is not supported. This is because the other ions in the haemolymph fluctuate considerably in changing salinities while the sodium to chloride ratio remains at unity. The maintenance of the haemolymph chloride concentration hypoionic to the medium at salinities above the isosmotic point reveals active excretion, whilst sodium is maintained at approximately the medium concentration. This would imply that the haemolymph sodium to chloride ratio was dependent upon the concentration of sodium in the haemolymph.

6.2.2.3 Osmotic pressure and sodium concentration (Fig.20)

If the haemolymph osmotic pressure and sodium concentration of the haemolymph are plotted against salinity, an interesting relationship appears. The two graphs follow each other very closely, with the exception of the values for freshwater. Here it would seem that the haemolymph osmotic pressure increases whilst the sodium concentration decreases. Although the two graphs are close they are not superimposed, with the osmotic pressure being slightly higher than sodium concentration at all salinities. If the osmotic pressure and sodium concentrations are expressed as percentages

(Fig.20) The relationship between HOP and haemolymph [Na] in adult prawns held at different salinities.(27°C).



of their concentration in full strength seawater, a direct comparison can be made. A two sample t-test on the arc-sin transformed data showed no significant difference between the two ($p<0.05$).

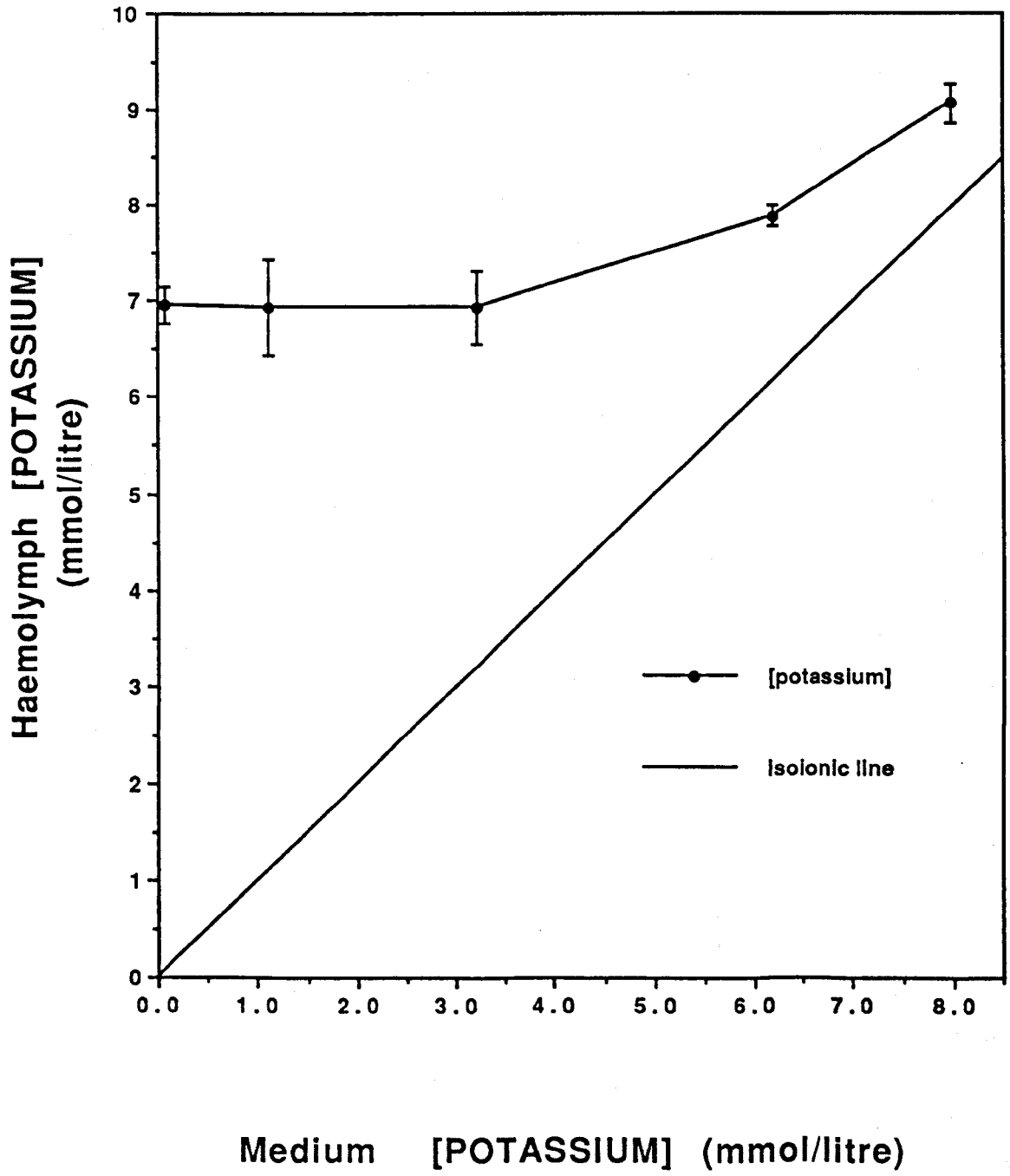
This apparent close relationship between osmotic pressure and sodium concentration is not altogether surprising in view of the fact that sodium chloride is thought to provide the major part of the osmotic pressure of the haemolymph. However, as was shown in section 6.2.2.2, the sodium chloride concentration does not account for all of the osmotic pressure. Thus it appears that the prawn regulates its haemolymph sodium concentration such that it is equivalent to the sodium concentration found in seawater of the same osmotic pressure. The haemolymph chloride concentration does not follow that of seawater at the same osmotic pressure. This lower chloride concentration has the effect of necessitating an alternative anion to maintain electroneutrality. This anion would electrically balance the chloride, magnesium, strontium and potassium in the haemolymph. If this is so then the concentration of this anion would need to be quite high ($\approx$17.5 $mmol.dm^{-3}$ if a divalent anion). Ahearn (1978) implies that this anion is sulphate. If this were the case it might explain the decrease in chloride concentration at high salinity, as it might be due to compensation for an elevated haemolymph sulphate concentration.

The close manner in which the haemolymph sodium concentration follows that of seawater having the same osmotic pressure as the prawns' haemolymph, suggests that the primary controlling factor in the regulation of the haemolymph sodium concentration (and hence chloride concentration, section 6.2.2.2) is the haemolymph osmotic pressure. Apart from sodium, the concentration of the other ions in the haemolymph bears no relation to those found in seawater of the same osmotic pressure.

6.2.2.4 Potassium (Fig. 21)

Potassium was hyperregulated at all the salinities tested with a slight rise in the potassium concentration in higher salinities. In freshwater and low salinities it appears that the haemolymph potassium was maintained at approximately the same concentration, there being no significant difference between the haemolymph potassium concentrations in 0-12^{o}/ooS seawater (two sample t-test, $p<0.05$). The range of

(Fig.21) Haemolymph [K] of adult prawns held at different salinities.



haemolymph potassium concentrations was between 6.93 - 9.07 mmol.dm^{-3}.

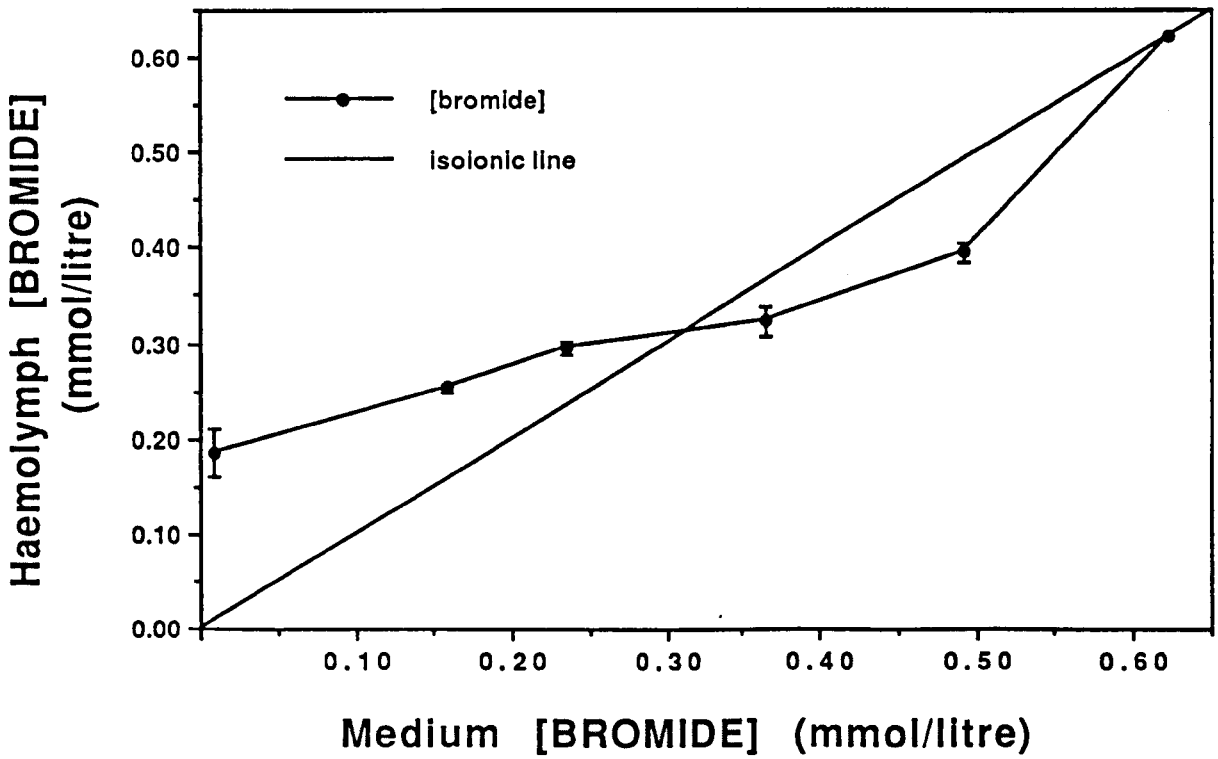
The hyperregulation of the haemolymph potassium concentration at all salinities tested (0-28 °/oo) contradicts the conclusion of Campbell & Jones (1989) (section 6.1.4), that the palaemonid prawns hyporegulate their haemolymph potassium concentrations in high salinities (Table XXIV). This is evident from the potassium regulation graph (Fig. 21) which shows the hyperregulation of the haemolymph potassium concentration. However, the concentration of potassium that the caridean prawns (section 6.1.4) maintain in their haemolymph, when in full strength seawater, was similar to that found in *Macrobrachium rosenbergii* in lower salinities and freshwater. It may be that a concentration of approximately 6.9 - 7.5 mmol.dm^{-3} is the most desirable for the correct functioning of Na/K ATPase in these species, or for the maintenance of the correct cell volume (Moran & Pierce 1984).

The gradual rise in haemolymph potassium concentration of *Macrobrachium rosenbergii,* when exposed to increased salinity, was also found by Stern *et al* (1987). This increased haemolymph potassium concentration should not be due to the increased sodium transport, since the haemolymph sodium concentration of the prawn conforms to the medium sodium concentration at these salinities. Therefore, this increase may simply be due to the elevated environmental concentration being too great for the prawns' potassium homeostatic mechanisms to cope. This would not be surprising, as in the freshwater environment the ionoregulatory mechanisms have to conserve ions rather than excrete excess.

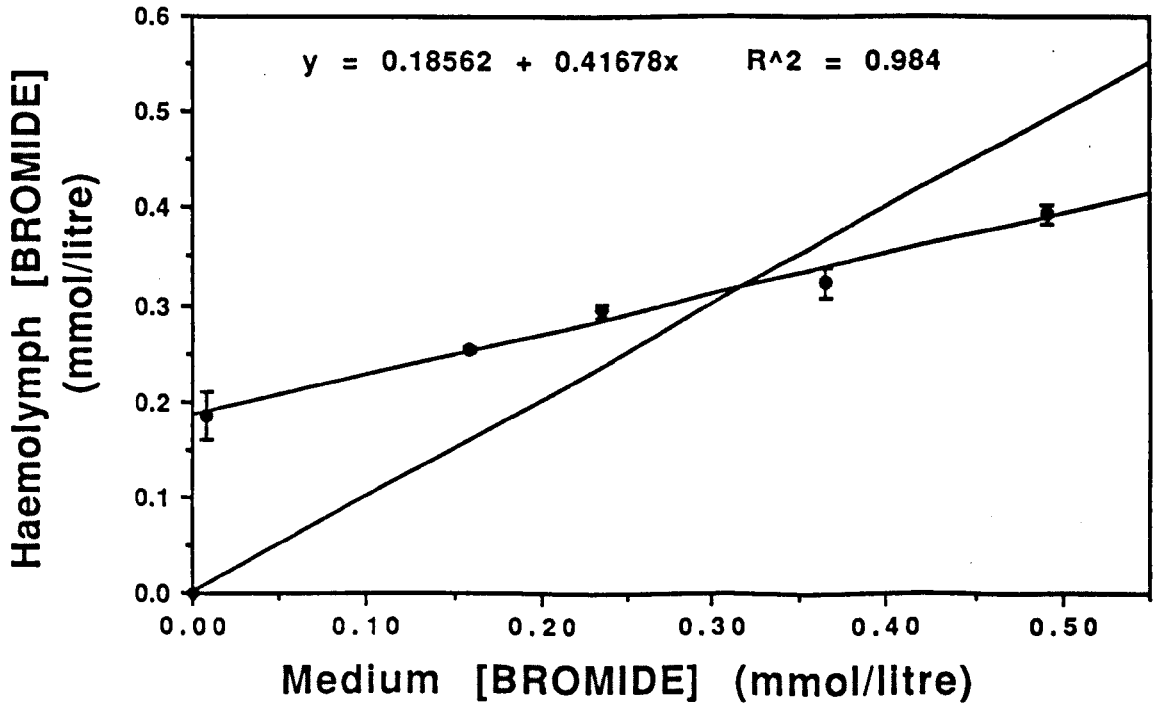
6.2.2.5 Bromide (Figs. 22,23)

The haemolymph bromide concentration was hyperregulated in low salinities up to the isoionic point. At medium bromide concentrations beyond the isoionic point bromide was hyporegulated, but this ceased at the highest salinity tested (25 °/oo). At this salinity the prawns' haemolymph bromide concentration conformed to the medium concentration. In freshwater, the haemolymph bromide concentration wa equivalent to that found in 7-8 °/ooS seawater (Fig. 22). There was a continuous and significant (two sample t-test, $p<0.05$) increase in the haemolymph bromide concentration with increasing salinity. This increase is an almost straight line relationship ($r= 0.984$),

(Fig.22) Haemolymph [Br] of adult prawns held at different salinities.



(Fig.23) The relationship between haemolymph and medium [Br] in adult prawns held at salinities up to 20 parts per thousand. A regression line is fitted to the data.



(Fig.23):

[haemolymph bromide] = 0.41678 [medium bromide] + 0.18562

The regulation of bromide might be taken as an indication that it has a specific function that requires it to be present in the haemolymph and tissues at a certain concentration. In freshwater, the prawn maintains a haemolymph bromide concentration equivalent to 7.5 $^{o}/_{oo}$S seawater (0.185 mmol.dm^{-3}). This further contributes to the notion that bromide plays a role in its physiology, as this represents a concentration factor of over 23 times the environmental concentration (freshwater ≈ 0.008 mmol.dm^{-3}).

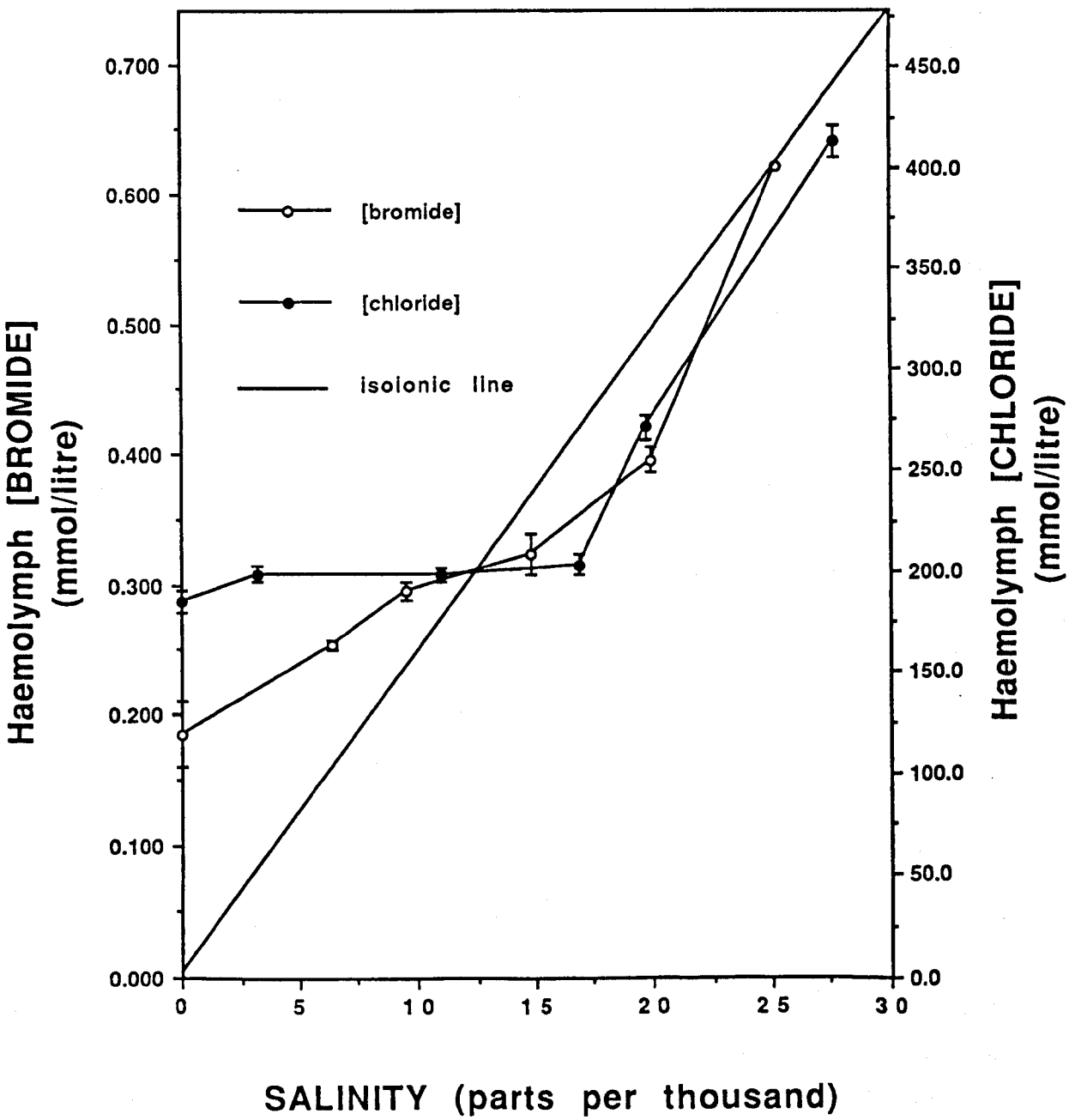
An alternative view is that lack of discrimination between bromide and chloride by the chloride uptake mechanism is giving this regulation pattern. If this were the case, then due to the relatively fixed ratios between chloride and bromide in seawater, there should be a corresponding fixed ratio between the two in the haemolymph. The possible relationship between chloride and bromide will be discussed in the next section (6.2.2.6).

6.2.2.6 Bromide and chloride (Fig.24).

This graph represents the haemolymph chloride and bromide concentrations plotted on different y-axes, against salinity. The scales of the y-axes are such that, for a given salinity on the x-axis, the y-axes will read the bromide and chloride concentrations found in seawater at that salinity.

Superimposing the regulation curves for bromide and chloride does not show a strong relationship between the two ions. At low salinity the haemolymph bromide concentration is much lower than the chloride concentration. As the salinity increases, the chloride is maintained at a relatively constant level whilst the bromide increases steadily. At the isoionic point for the two ions their concentrations are the same as those in seawater. At salinities higher than the isoionic point the two ions are in approximately the same ratio up to the highest salinity. At this salinity the haemolymph bromide concentration is the same as that of the medium, whilst the chloride concentration is still maintained hypoionically.

(Fig.24) The relationship between haemolymph [Cl] and [Br] in adult prawns held at different salinities. The common isoionic line is also shown.



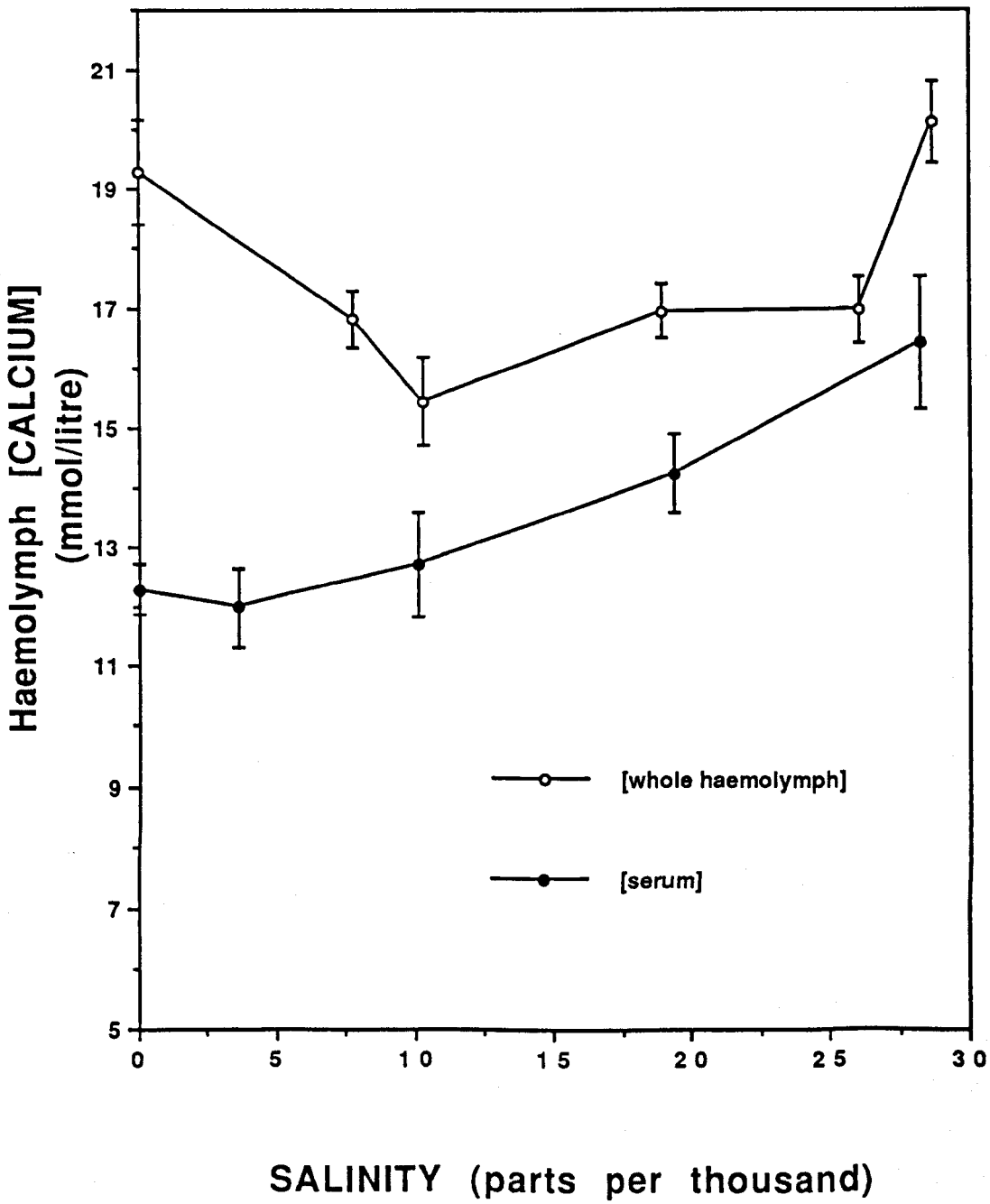
If, as suggested in section 6.2.2.5, the regulation of bromide is purely due to lack of discrimination by the chloride transport mechanism, it should be possible to superimpose the two graphs. This is because the fixed ratio between the two ions in seawater should be reflected in the haemolymph as well. However, this does not mean that the haemolymph concentrations will reflect the seawater concentrations, but does imply that the ratio between the two ions should be fixed over a range of salinities. It seems that, whilst both ions are regulated in dilute salinity, chloride is regulated more strongly. The maintenance of an almost constant chloride concentration in the haemolymph is in contrast to the gradual increase in the concentration of bromide. This discounts the idea that the chloride transport mechanism does not discriminate between the two ions.

A possible explanation for the increase in haemolymph bromide whilst the chloride remains constant might be that the chloride uptake mechanism has different affinities for the two ions. Thus as the bromide concentration in the medium increases, bromide is transported preferentially. The almost straight line relationship between medium and haemolymph bromide concentrations at the five lowest salinities suggests that there might be specific mechanisms for the uptake and excretion of bromide. At high salinity, chloride is actively hyporegulated and this seems to be due to its close relationship with sodium (section 6.2.2.2). However, the haemolymph bromide concentration at this salinity is isoionic with the medium. This is an indication that the prawn is no longer able to control its haemolymph bromide concentration by excretion. This failure to regulate the haemolymph bromide concentration at a salinity at which the prawn is still able to regulate its haemolymph chloride concentration implies that the mechanism for the excretion of the two ions might be discriminatory.

6.2.2.7 Calcium (Fig.25).

The serum calcium concentration showed a gradual increase with increasing salinity (12.3-16.4 $mmol.dm^{-3}$). These calcium concentrations were hyperionic to the medium at all salinities. The serum calcium concentrations of prawns in freshwater (12.3 $mmol.dm^{-3}$) were greater than the calcium concentration of full strength seawater (10.5 $mmol.dm^{-3}$).

(Fig.25) Whole haemolymph and serum [Ca] of adult prawns held at different salinities.



The whole haemolymph calcium concentrations showed a decrease in the calcium concentration with increasing salinity. The whole haemolymph calcium concentration was approximately the same (≈15.5-17 $mmol.dm^{-3}$, no significant difference, two sample t-test, $p<0.05$) at salinities between 7.7-26.0 °/oo, after which there was a sharp increase to a concentration of 20 $mmol.dm^{-3}$.

The increase in the serum calcium concentration with increasing salinity is somewhat strange as the prawn is actively importing calcium since there is no diffusion gradient from the medium. There are several possible reasons for this increasing calcium concentration, one or all of which might be occurring. Firstly, the increasing calcium is for the maintenance of haemolymph electroneutrality, compensating for a rise in serum sulphate. This is logical, as magnesium is maintained at low concentration and could not be used, and calcium is an alternative divalent cation. Secondly, the calcium might be acting as a counter-ion for the excretion of magnesium. Magnesium is five times more abundant in seawater than calcium, yet its haemolymph concentration is very low (1.7-2.4 $mmol.dm^{-3}$, section 6.2.2.8), thus continual excretion is necessary to counter diffusive influx. It may be that, in the same way that potassium is hyperregulated as a counter-ion for sodium regulation, calcium is performing the same function for magnesium.

The calcium concentrations in whole haemolymph are regulated in a more obscure manner. It is considered that part of the calcium component of haemolymph is bound in some way, and this is apparent from the higher concentrations found in whole haemolymph relative to the serum. The total calcium concentration in whole haemolymph at salinities between 7.7-26 °/oo is relatively stable, and this implies that the bound component is reduced in compensation for the increased concentration found in the serum. This steady concentration might be desirable for the reasons suggested in sections 6.1.6 and 8.1, due to the effect of calcium on various physiological processes.

The serum calcium concentrations in the various salinities (12.3-16.4 $mmol.dm^{-3}$) lie within the same range as those listed for other palaemonid species (12.3-17.7 $mmol.dm^{-3}$, section 6.1.2, Table XXIV). However, these other species were all maintained in full strength seawater, whereas the maximum concentration of calcium *Macrobrachium rosenbergii* was exposed to was equivalent to 26 °/ooS. Stern *et al.* (1987) reported similar values for *Macrobrachium rosenbergii* and Moreira *et al.* (1988) for *Macrobrachium carcinus*, held in freshwater, seawater and various saline

(≈12.5-17.5 mmol.dm^{-3} and 10.5-12.5 mmol.dm^{-3}, respectively). It seems that this range of serum calcium concentrations are optimal for these palaemonid prawns irrespective of the salinity of the medium.

6.2.2.8 Magnesium (Fig. 26)

The serum concentration of magnesium increased from the lowest concentration of 1.675 mmol.dm^{-3} in freshwater, to a relatively stable value of 2.0-2.4 mmol.dm^{-3} in salinities between 3-21 °/oo (no significant difference, two sample t-test, $p<0.05$). At a salinity of 27 °/oo the concentration increased sharply. This was approximately the same salinity at which the total calcium concentration also increased sharply (28.7 °/oo).

The magnesium concentration in whole haemolymph gives a more confusing picture. The concentrations were lower than those found in the serum. This suggests that there is no bound magnesium in the haemolymph, and that the non serum components of the haemolymph might exclude magnesium. However, these differences might be attributable to analytical variation caused by the use of serum and acid digested blood. Interestingly, the concentrations for serum and whole haemolymph for prawns in freshwater were similar (1.675 and 1.636 mmoldm^{-3}, respectively, no significant difference, two sample t-test, $p<0.05$).

Table (XXVII) Haemolymph magnesium concentrations in various crustaceans - Adapted from Walters & Uglow (1981), Mantel & Farmer (1983) and Tentori & Lockwood (1990).

FRESHWATER OR LOW SALINITY ACCLIMATED SPECIES	mmol.dm^{-3}	
Triops longicaudatus	0.9	Horne (1966)
Branchinella australensis	0.46	Geddes (1973)
Limnocalanus macrurus	1.25	Bayley (1969)
Austropotamobius pallipes	0.5-4.2	Bryan (1967),Riegel (1968) Greenaway (1974)
Orconectes limosus	2.8	Andrews (1967)
Orconectes rusticus	1.2	Sharma (1968)
Sudanonautes africanus	10.6	Lutz (1969)
Paratelphusa hydrodromus	7.8	Ramamurthi (1967)

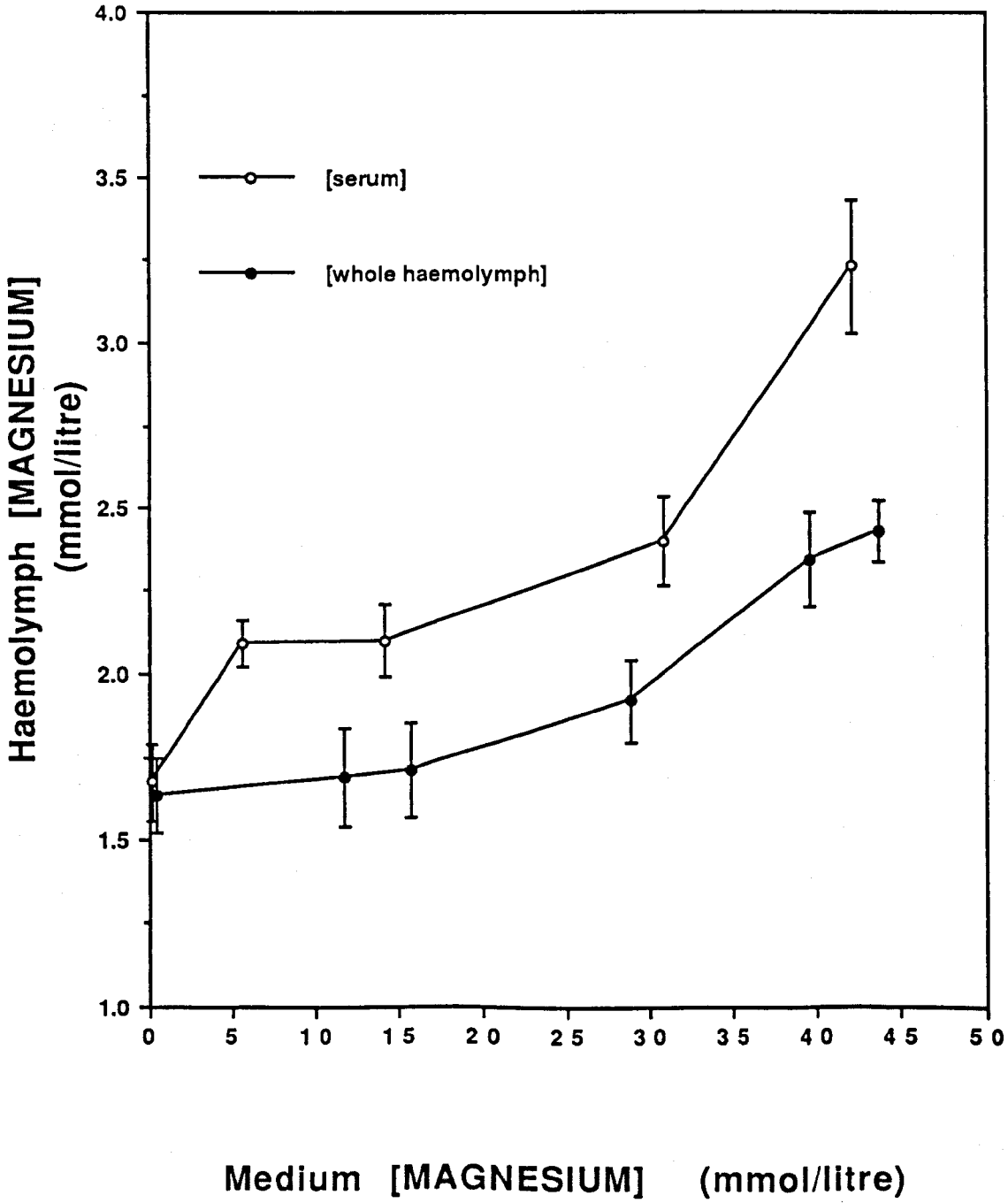
FRESHWATER OR LOW SALINITY ACCLIMATED SPECIES

	$mmol.dm^{-3}$	
Holthuisana transversa	4.7	Geenaway & Macmillan (1978)
Eriocheir sinensis	3.3	DeLeersnyder (1967a)
Macrobrachium rosenbergii	2.0	Stern *et al.* (1987)
Macrobrachium rosenbergii	0.79	Fieber & Lutz (1985)
Pacifastacus leniusculus	2.73	Wheatley & McMahon (1982)

MARINE OR BRACKISHWATER SPECIES IN 35°/oo SEAWATER.

	$mmol.dm^{-3}$	
Ligia oceanica	20.0	Parry (1953)
Penaeus aztecus	24.0	Venkatamariah *et al.* (1974)
Trachypenaeus similis	21.0	McFarland & Lee (1963)
Sicyonia dorsalis	43.0	McFarland & Lee (1963)
Pandalus hypsinotus	37.0	Mackay & Prosser (1970)
Palaemon serratus	13.0	Parry (1954)
Crangon crangon	2.3-8.8	Hagerman (1971,1973a,1978)
Macrobrachium olfersii	2.59	McNamara *et al.* (1990)
Nephrops norvegicus	10.0	Robertson (1961)
Homarus gammarus	10.0	Glynn (1968)
Homarus americanus	7.0	Prosser (1973)
Panulirus argus	16.0	Malley (1977)
Panulirus longipes	13.5	Dall (1974a)
Paralithodes camtschatica	37.0	Mackay & Prosser (1970)
Coenobita perlatus	43.0	Gross & Holland (1960)
Carcinus maenas	16.0	Walters & Uglow (1981)
Carcinus mediterraneus	12.0	Lucu (1969)
Cancer magister	12.0	Hunter & Rudy (1975)
Cancer pagurus	32.9	Walters & Uglow (1981)
Chionoecetes tanneri	45.0	MacKay & Prosser (1970)
Pugettia producta	46.0	Cornell (1979)
Cardisoma armatum	10.0	DeLeersnyder & Hoestlandt (1964)
Maia squinado	39.1	Walters & Uglow (1981)
Eriocheir sinensis	12.0	DeLeersnyder (1967a)
Goniopsis cruentata	12.0	Zanders (1978)
Hemigrapsus edwardsi	13.8	Bedford & Leader (1977,1978)
Hemigrapsus nudus	17.0	Dehnel (1966)
Hemigrapsus oregonensis	18.0	Dehnel & Carefoot (1965)
Pachygrapsus crassipes	9.0	Gross (1959);Prosser *et al.* (1955)
Uca crenulata	30.0	Gross (1964b)
Uca pugilator	10.0	Wright *et al.* (1984)
Uca minax	10.0	Wright *et al.* (1984)
Uca pugnax	10.0	Wright *et al.* (1984)
Callianassa jamaicense	50.0	Felder (1978)
Pagurus bernhardus	23.0	Walters & Uglow (1981)
Corystes cassivelaunus	35.0	Walters & Uglow (1981)
Acanthephyra sexspinosa	8.9	Tentori & Lockwood (1990)
Plesionika rossignoli	13.8	Tentori & Lockwood (1990)
Gennadas brevirostris	15.0	Tentori & Lockwood (1990)

(Fig.26) Whole haemolymph and serum [Mg] of adult prawns held at different salinities.



the results somewhat, although variation within groups suggested that this was not particularly significant.

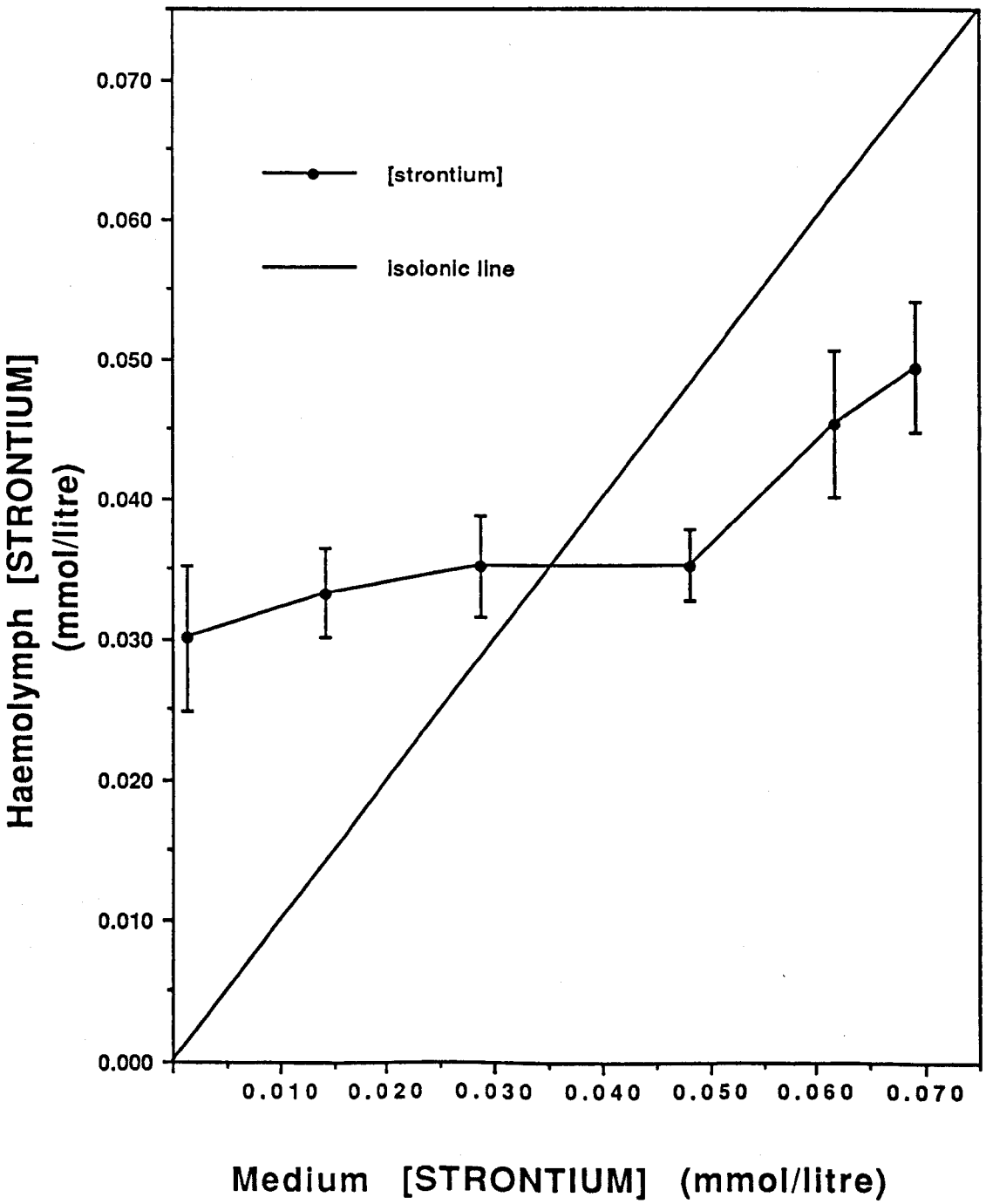
Fig. 27 shows that strontium was regulated strongly over a salinity range between freshwater and 18°/ooS. At higher salinities, the haemolymph strontium concentration increased, but was still maintained hypoionic to the medium.

The regulation of strontium within the range of concentrations found in seawater is not surprising. If the transport mechanisms for calcium and magnesium distinguish poorly between these ions then it might be expected that strontium was regulated as a function of its ratio to the other ions in the external medium. If it is assumed that the influx of magnesium to *Macrobrachium rosenbergii* in seawater is a relatively passive process, then the haemolymph concentration is controlled by the rate of excretion. This cannot be the case with strontium since it is hyperregulated in salinities below 13-14°/oo seawater. Thus the inference is that the ion is being concentrated from the medium by an actively operating uptake mechanism. If this is happening, then it must be due to the lack of specificity of the calcium uptake mechanism. This is because calcium is maintained hyperionic to the medium at all salinities tested and is therefore continually being actively absorbed from the environment. Due to their proximity in the periodic table, the similarity in ionic radii between calcium and strontium is greater than that between strontium and magnesium (ionic radii: Mg=0.66, Ca=0.99, Sr=1.12 Å, Dodd 1967). In this case it might be expected that haemolymph strontium concentrations would be a function of the strontium : calcium ratio. This would be so, unless the excretory mechanisms for strontium and calcium were different. As calcium concentration is maintained hyperionic to the medium in all salinities, then active excretion would seem to be unneccessary. The result of the lack of excretion of calcium would be that strontium would be taken up with calcium, and not excreted. The expected relationship between haemolymph strontium concentration and salinity would therefore be linear.

The regulation of haemolymph strontium concentration below that of the medium at salinities above 13-14°/oo suggests that an active excretion mechanism exists for strontium. Thus strontium is both actively absorbed and excreted in the freshwater prawn in different salinities.

It is possible that uptake of strontium is via the calcium absorbtion

(Fig.27) Haemolymph [Sr] of adult prawns held at different salinities.



mechanism, and excretion via the magnesium excretion mechanism. This is effectively disproven by the haemolymph strontium concentration of prawns held in freshwater. This concentration is only slightly less than that at which the prawns regulate the haemolymph when in dilute seawater. This implies that the concentration of strontium in the haemolymph might be critical to the prawn, as the ratios between strontium, magnesium and calcium found in seawater, do not exist in freshwater.

6.2.2.10 Strontium, magnesium and calcium (Figs. 28,29).

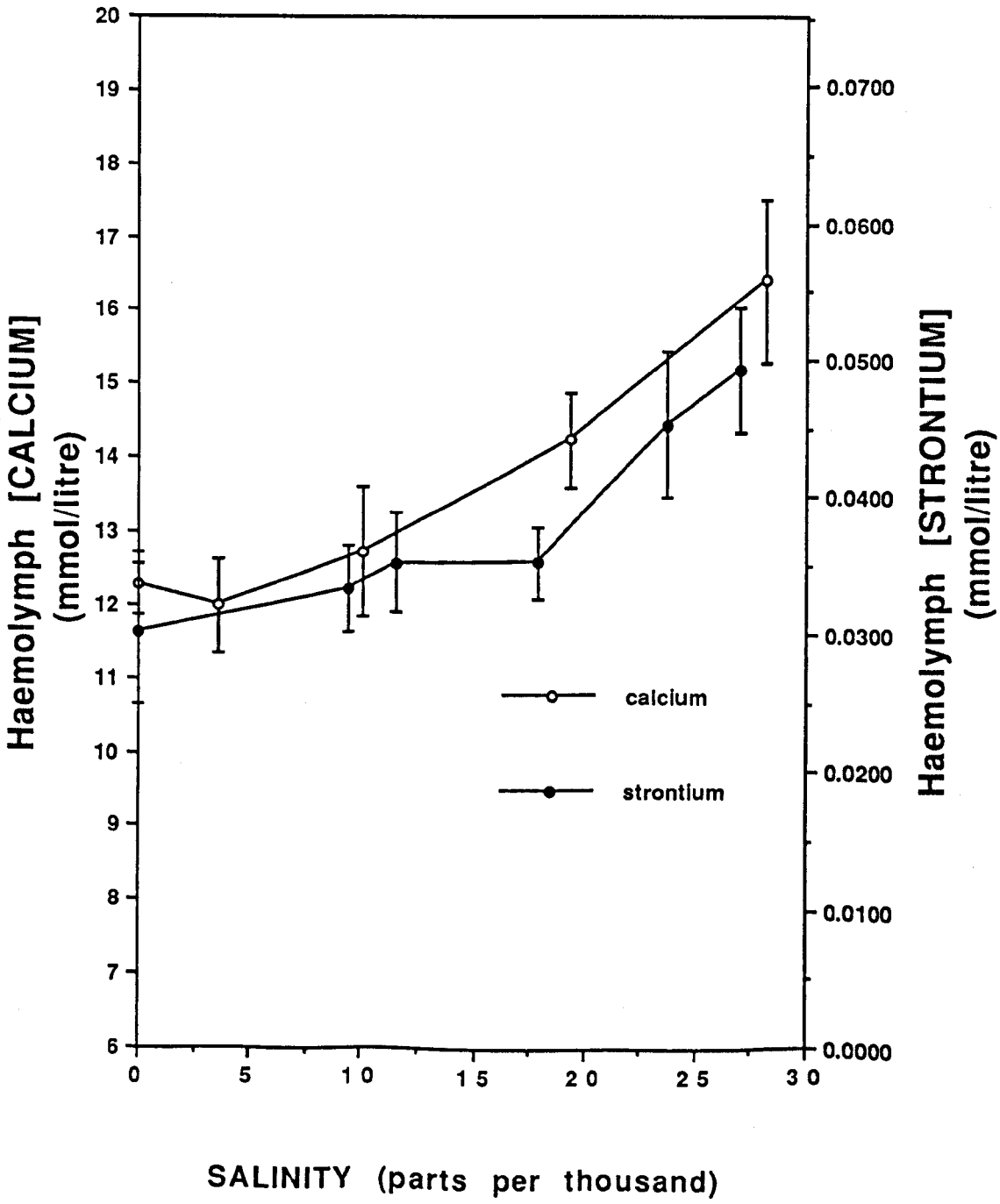
In order to establish whether there was any perceptible relationship between the regulation of strontium, calcium and magnesium, the graphs showing the haemolymph regulation of these ions in varying salinities were superimposed. To plot these graphs on the same axes would be pointless due to the wide difference in their haemolymph concentrations. If a direct relationship exists then there should be a simple multiplication coefficient between the concentrations of the ions.

Calcium and strontium can also be superimposed, but the axes of the two graphs do not begin at zero (Fig. 28). This means that there is not a direct ratio between them, and as such they are probably unrelated.

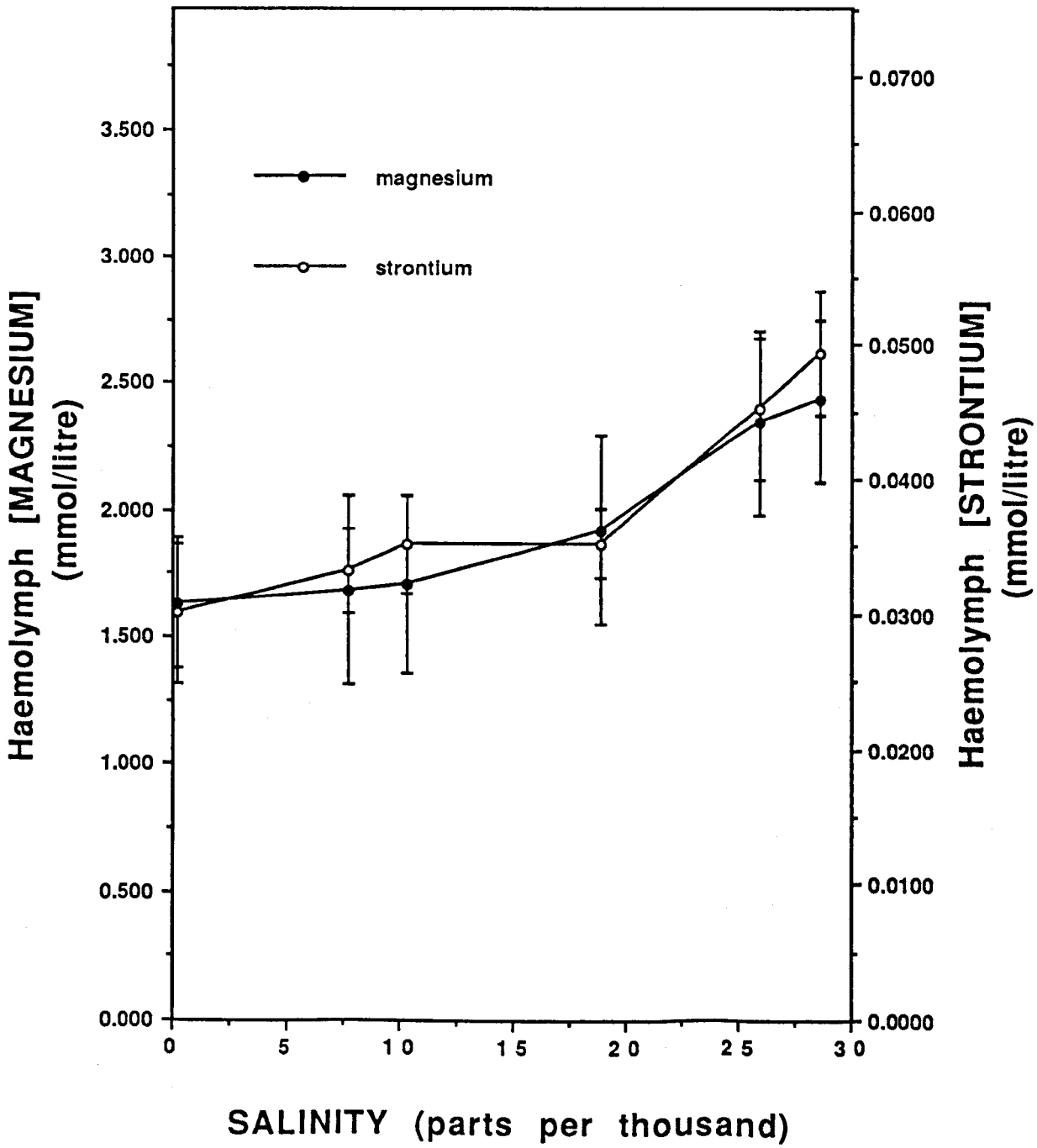
The graph of strontium and magnesium shows a strikingly close relationship between these ions (Fig.29). This is unusual since strontium and magnesium have dissimilar ionic radii, indeed both are more closely related to calcium (section 7.2.2). The magnesium/strontium graph shows a direct and very close ratio between the two ions (1:53). This close ratio between these two ions is also maintained in freshwater. This is significant as the concentrations of these ions in freshwater are not in a fixed ratio as they are in seawater. If the regulation of these two ions was unrelated then this would be expected to be manifested as different ratios in the haemolymph of prawns held in freshwater from those held in seawater.

The close ratio between the haemolymph strontium and magnesium concentrations is maintained even in high salinity. This is the point where the regulation of strontium appears to deteriorate. If the regulation processes of these two ions were unrelated it might be expected that the strong regulation of magnesium would be unaffected

(Fig.28) The relationship between haemolymph [Ca] and [Sr] of adult prawns held at different salinities.



(Fig.29) The relationship between haemolymph [Mg] and [Sr] of adult prawns held at different salinities.



by the change in strontium concentration. Whilst the haemolymph magnesium concentration does increase slightly in increasing salinity it is only as a small percentage of the external medium concentration, whereas the increase in the haemolymph strontium concentration is far greater. This raises the question as to why the haemolymph magnesium concentration increases at all, since it is maintained so far below the medium concentration at salinities between 0-28 $^{o}/_{oo}$?

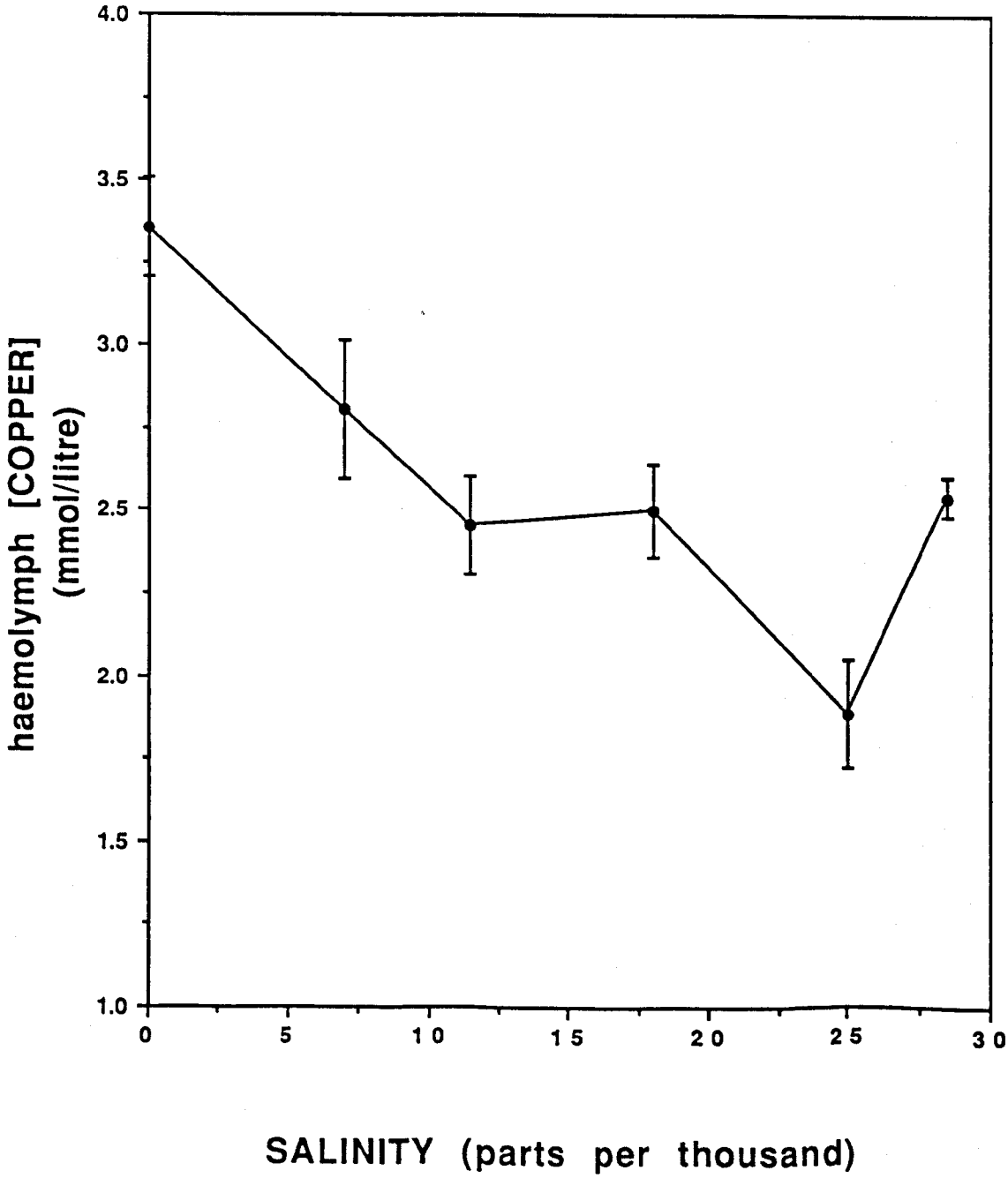
The regulation of strontium at a relatively high concentration (equivalent to 14$^{o}/_{oo}$S seawater) over a range of salinities (0-14$^{o}/_{oo}$) implies that it is essential for some physiological process. The close relationship with magnesium suggests that perhaps the low magnesium concentrations of the haemolymph are due to the regulation of strontium. If this is the case (and not *vice versa*), then the observations upon the effect of magnesium on nervous systems may have been due to an imbalance in strontium regulation. It is also possible that contamination of calcium and magnesium salts with strontium may have affected the results of this work. This is a possible, although tenuous, explanation of the conflicting results from Zanders (1980) and Bethe (1929 quoted in Robertson 1953).

The concept of a role for strontium in the physiology of *Macrobrachium rosenbergii* is an attractive one. The low concentration found in both the environment and the haemolymph means that its regulation is probably quite sensitive, and this sensitivity would have the effect of enabling a change in the concentration in the haemolymph without significantly disturbing the osmotic pressure or other ionic concentrations. The work of Bidwell *et al.* (1986,1990) suggests a specific role in a biochemical pathway relating to the calcification process in the embryos of the mollusc *Aplysia californica*.

6.2.2.11 Copper (Fig. 30)

The haemolymph copper concentration decreased from a maximum value in freshwater (3.354 mmol.dm^{-3}), to a concentration of 2.454 mmol.dm^{-3} with increasing salinity. This value was approximately constant over a range of salinities (11.5-28.5$^{o}/_{oo}$, no significant difference, except at 25$^{o}/_{oo}$S, two sample t-test, $p<0.05$). However, the haemolymph copper concentration of prawns held at 25$^{o}/_{oo}$S was much lower (1.886 mmol.dm^{-3}) than that at higher or lower salinities. The range of

(Fig.30) Haemolymph [Cu] of adult prawns held at different salinities.



copper concentrations in the haemolymph of *Macrobrachium rosenbergii* is considerably higher than those reported for other species (Table XXVIII).

Table (XXVIII) Haemolymph copper concentrations of various decapod crustacea.

species	average	range	reference
		($mmol.dm^{-3}$)	
Carcinus maenas	1.392	0.597-1.405	Kerkut *et al.* (1961)
	-	0.227-1.263	Spaargaren (1983)
	-	0.896-1.086	Bjerregaard & Vislie (1986)
Crangon crangon	-	0.645-2.510	Djangmah and Grove (1970)
Austropotamobius pallipes	0.482	0.090-0.888	Bryan (1967)
Eriocheir sinensis	0.275	-	seawater,Schoffeniels (1976)
	0.559	-	freshwater,Schoffeniels (1976)
Macrobrachium rosenbergii	-	1.886-3.354	This work (0-28 ^{o}/ooS)

It is generally assumed that the copper content of the haemolymph is almost entirely present as haemocyanin (Kerkut *et al.* 1961; Djangmah & Grove 1970; Jeuniaux 1971; Smith & Dall 1982), and thus the very high copper concentrations found in the haemolymph of *Macrobrachium rosenbergii* reflects a high concentration of haemocyanin. This high concentration might be due to its contribution to the colloid osmotic pressure of the haemolymph. In section (6.2.2.2) it was suggested that there was a considerable shortfall in the osmotic pressure contributed by sodium chloride and that measured in the haemolymph of *Macrobrachium rosenbergii.* This would indicate the requirement for other ions and organic molecules in the haemolymph to provide an additional osmotic pressure to that provided by sodium chloride. If this is the case then haemocyanin is the most likely form of the organic fraction, and this would explain the high haemolymph concentration relative to the marine species listed (Table XXVIII) It does not explain why the haemolymph sodium concentration is the same as would be found in seawater of the same osmotic concentration.

There is a reduction in the haemolymph copper concentration from freshwater to a salinity of 12^{o}/oo of approximately 27%. This might be partly explained by an increase in the blood volume. The saturation concentration of oxygen in water declines with increasing salinity, therefore the decrease in haemolymph copper cannot be

attributed to a reduction in the requirement for respiratory pigment. However, Schoffeniels (1976) suggests that there is a higher tissue demand for oxygen when *Eriocheir sinensis* is in freshwater and thus it would be desirable to maintain a higher haemolymph concentration of haemocyanin. Schoffeniels (1976) also suggests that haemocyanin production might be a method of storing nitrogenous compounds with little thermodynamic activity. An increasing salinity from freshwater does mean that the requirement for haemolymph protein to maintain osmotic pressure is reduced. This would explain the observation that prawns maintained in higher salinity treatments had less viscous haemolymph than those in freshwater. The haemolymph was easier to sample and did not gel as rapidly in the prawns maintained in high salinity. Tan & Choong (1981) found a reduction in the total ninhydrin positive substances (NPS) in the haemolymph of *Macrobrachium rosenbergii,* when exposed to increasing salinity between 0-7.5^{o}/oo. At higher salinities (7.5-30^{o}/oo) there was no significant change in the NPS concentration. This is in good agreement with the changes in haemolymph copper concentrations of prawns exposed to varying salinity found in this work.

6.3 Effect of moult cycle on haemolymph ionic concentration in adult *Macrobrachium rosenbergii.*

The aim of this experiment was to try to establish whether bromide and strontium were important in the moult cycle in *Macrobrachium rosenbergii.* The reason for this was the implication of bromide in the formation of crustacean cuticles, and the apparent requirement for bromide by larval prawns (sections 5.2, 5.4, 7.5.6). It was thought that if significant fluxes in the haemolymph bromide concentration occurred over the moult cycle this might imply a role in the formation of the cuticle, sclerotisation or functioning of moulting enzymes. Strontium was studied as it was found at quite high concentration in the adult prawns when in freshwater (section 6.2.2.9). It was thought that this might be due to some role in either the calcification process or enzyme function. If it had no real function it might be expected that its regulation would follow that of either magnesium or calcium.

6.3.1 Materials and methods

Adult prawns were maintained in recirculating system 3 according to the methods described in section 3.2. Haemolymph from eight adult prawns was sampled at five stages of the intermoult cycle. The stages sampled and their determination is detailed in section 3.3. Two series of samples were obtained, the first were used for analysis of Ca, Mg, Sr and Cu, the second for neutron activation analysis of Br.

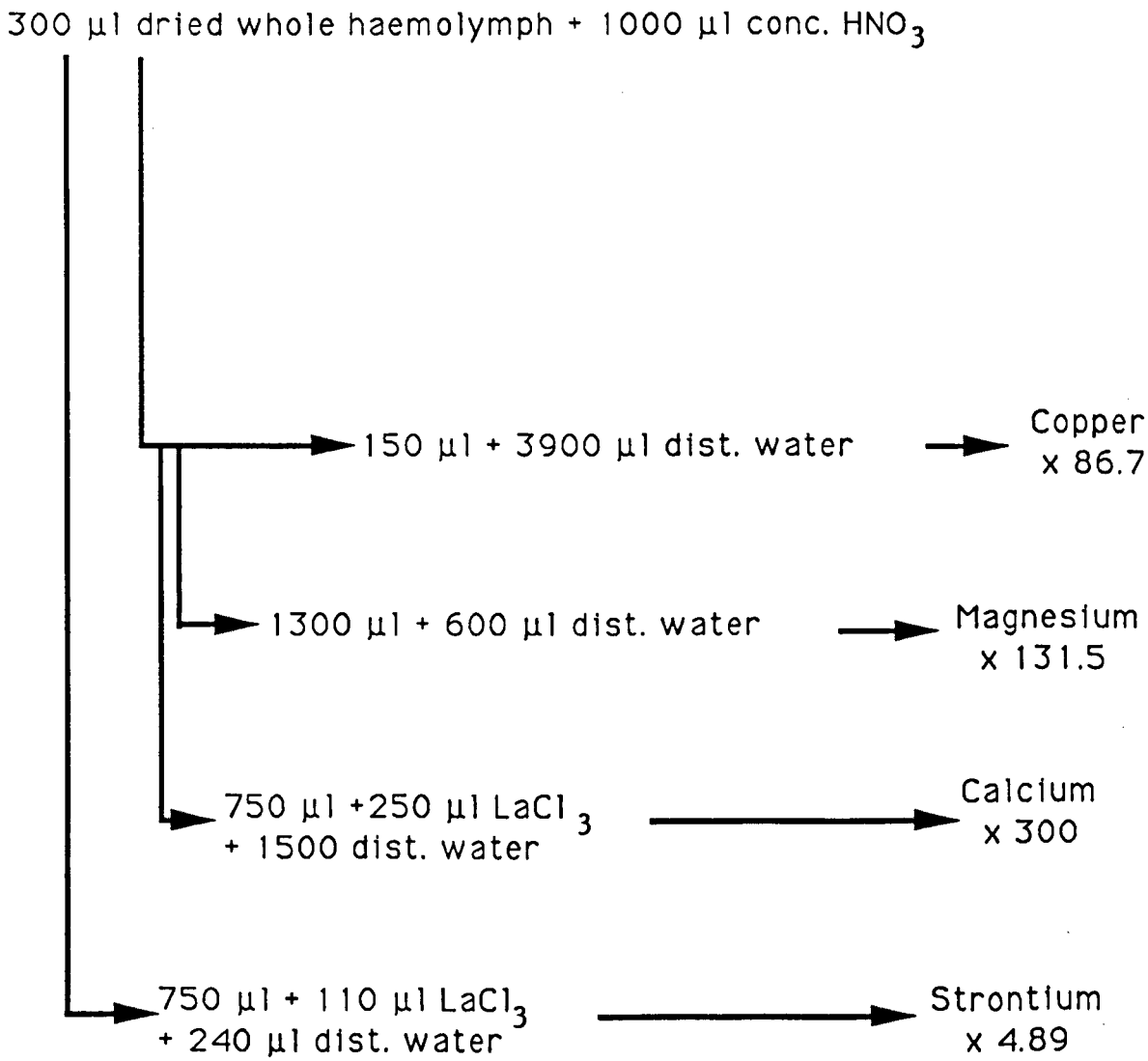
There were several reasons for using whole haemolymph for these analyses. One of these was the expectation of low strontium concentrations. To have used serum would not have provided sufficient sample volume for one reading of strontium using AAS, and it would not have been possible to analyse the other ions. A second reason was that the copper in the haemolymph is bound into haemocyanin and the use of serum would not have yielded an accurate representation of the total haemolymph copper. Calcium and magnesium are known to exist both as free and bound forms (Section 6.1.6) and hence total concentrations are possibly more reliable indicators of haemolymph changes over the moult cycle, although the relative proportions of the two forms probably changes.

The use of whole haemolymph provided sample volumes of approximately 300 µl. These samples were weighed, dried (60°C, 48 hours), reweighed and then digested in 'Aristar' concentrated nitric acid prior to dilution and analysis using AAS. The digestion and dilution protocol is given in Fig.31. The second set of samples for bromine/bromide NAA were weighed, dried (60°C, 48 hours) and reweighed. Analysis using NAA was performed at the Imperial College Reactor Centre as detailed in section 3.3.6.2. The intermoult bromine/bromide concentrations were analysed previously at SURRC. This gives some concern as to the consistency of the results, although the same standard solution was used for both analyses. This acts as an internal reference, as all bromide concentrations were calculated relative to this standard.

6.3.2 Results and discussion

All graphs were plotted as means ± the standard error of the mean. Where stated, two sample t-tests were performed using 'Statgraphics', a computer statistical

(Fig.31) Digestion and dilution protocol for whole blood analysis of prawns sampled over the moult cycle.



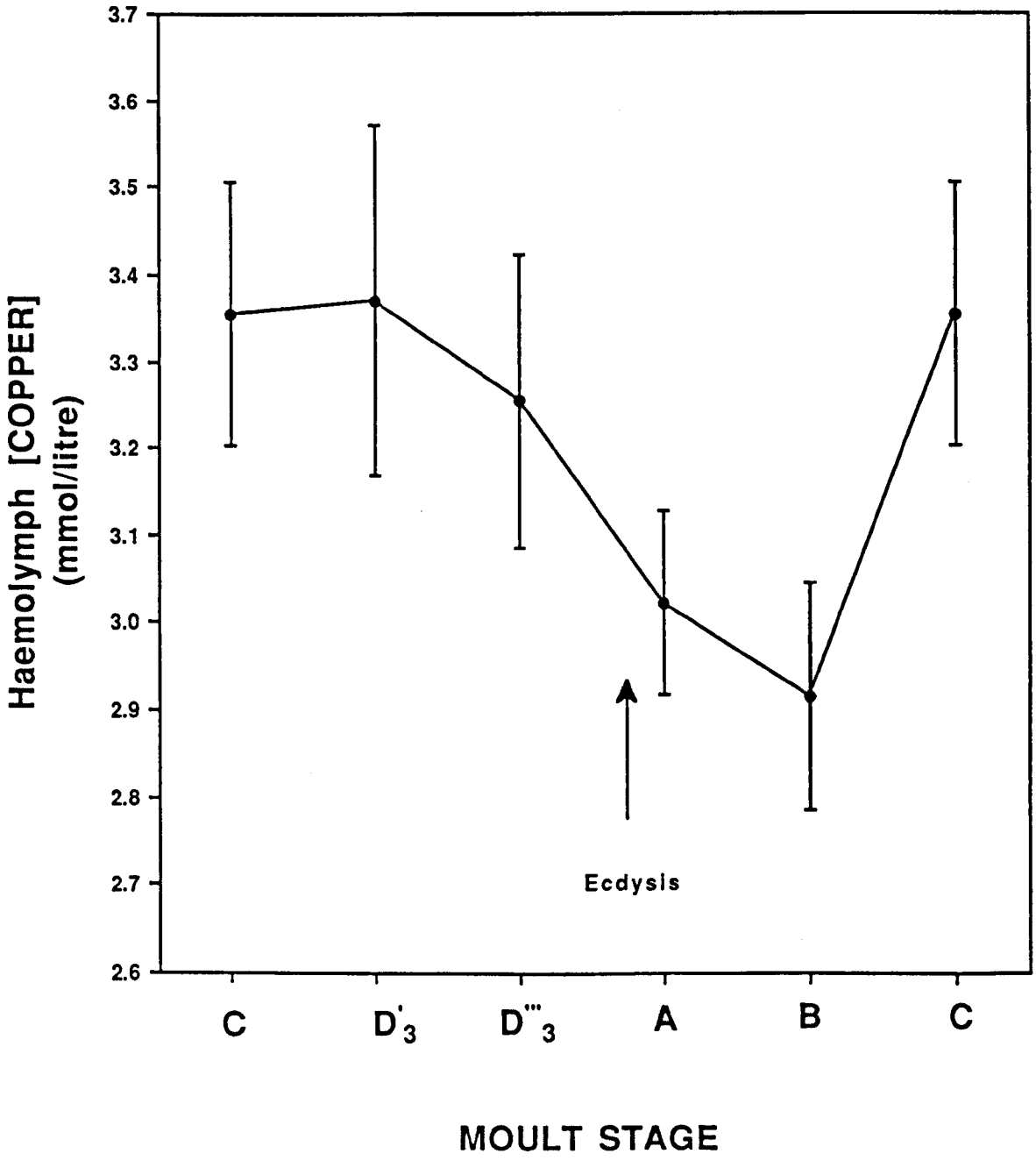
package.

6.3.2.1 Copper (Fig.32)

The haemolymph copper concentration declined from an intermoult level of 3.35 $mmoldm^{-3}$ to 2.92 $mmoldm^{-3}$ at moult stage B. This reduction was not statistically significant due to the wide range in the data (two sample t-test, $p<0.05$). This trend towards a reduction in the haemolymph copper concentration during and after the moult is in agreement with Kerkut *et al.* (1961) and Djangmah and Grove (1970), who suggested that the haemocyanin concentration of the haemolymph is highest during premoult, and is reduced at the time of the moult to a minimum concentration during stage B. Crustacean haemolymph osmotic pressure is increased prior to moulting (Baumberger & Olmsted 1928; Robertson 1937). This was observed in this work as an increase in the percentage dry matter in the haemolymph, indicating a concentration of the haemolymph. At the onset of moulting the haemolymph osmotic pressure decreases as water is absorbed. Thus, the reduction in copper concentration immediately premoult and until stage B, might be due to an increased requirement for cellular free amino acids to compensate for the higher haemolymph osmotic pressure. When crustaceans are transferred to a more dilute medium, a reduction in haemolymph osmotic pressure occurs, and this is accompanied by an increase in the haemolymph copper concentration. This is thought to be due to the loss of free amino acids from the tissues to the haemolymph, where they are incorporated into haemocyanin molecules (Schoffeniels 1976). The loss of free amino acids by the tissues is the cellular response to a decrease in the haemolymph osmotic pressure, and maintains cell volume. During the late premoult stage, as the haemolymph osmotic pressure increases, haemocyanin might be being broken down to provide free amino acids for cellular volume regulation. This does not explain why the haemolymph copper decreases after the moult, when the haemolymph osmotic concentration is reduced. Robertson (1960) suggests that dilution of the ionic content of the haemolymph occurs during postmoult due to the uptake of water. This would explain the reductions in the copper, strontium and calcium concentrations. It does not explain why magnesium is maintained at a constant concentration.

An interesting observation made during the sampling of prawns was that the colour of the haemolymph varied according to moult stage. Intermoult post-branchial haemolymph is almost colourless in prawns sampled from freshwater. This is in

(Fig.32) Variation in the haemolymph [Cu] of adult prawns over the moult cycle. Prawns held in freshwater at 27 °C.



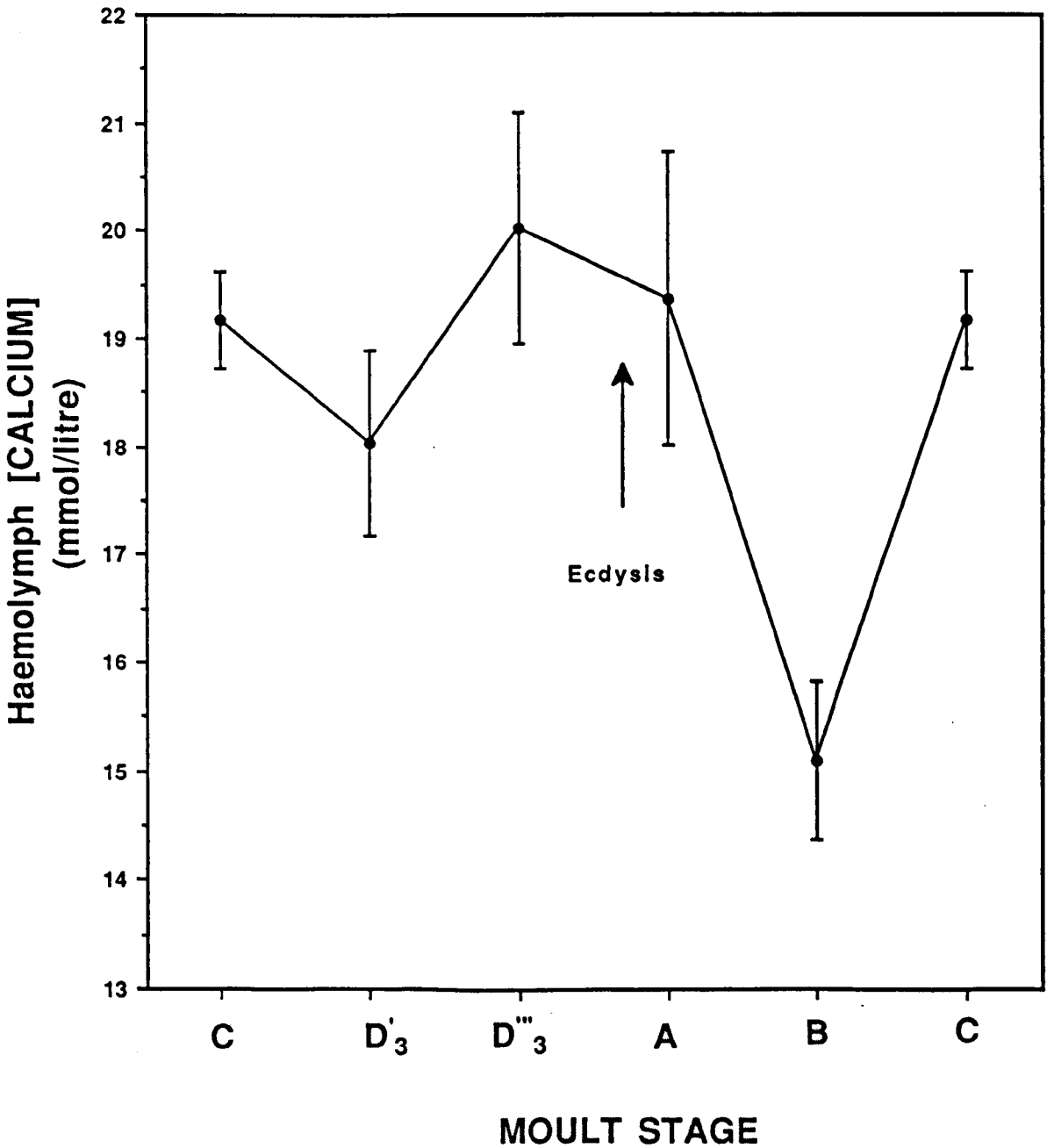
agreement with the O_2 concentration measurements carried out in section 8.3.5. The haemolymph from late premoult and early postmoult prawns appeared to be quite blue when sampled. This suggests that the prawn is increasing the oxygen saturation of its haemolymph, possibly as a compensation for the reduced haemocyanin concentration that occurs during the moult and postmoult stages, or as a result of changing haemolymph ionic concentrations affecting the affinity of the haemocyanin (section 8.1).

6.3.2.2 Calcium (Fig.33).

The haemolymph calcium regulation during the moult cycle does not show a distinct pattern. There was a slight increase in the calcium concentration between stages D_3' and D_3''', followed by a slight decrease (no significant difference, two sample t-test, $p<0.05$) at stage A. After stage A there was a significant decrease in the haemolymph calcium concentration (two sample t-test, $p<0.05$) to a minimum calcium concentration in stage B. The intermoult calcium concentration appeared to be slightly higher than that found at stage D_3' (no significant difference, two sample t-test, $p<0.05$), and this might be attributable to a reduction in blood volume. The minimum value found in stage B might have been due to the incorporation of calcium into the cuticle. Calcification commences at the end of stage A, and is concluded by the end of stage B. Full hardness of the cuticle is not achieved until approximately five days postmoult.

The decrease in the haemolymph calcium concentration in *Macrobrachium rosenbergii*. was also found by Fieber & Lutz (1982). These authors reported substantially lower calcium concentrations than those in the present study. Their figures are also in conflict with the intermoult calcium concentrations reported by Singh (1977) and Stern *et al.* (1987). Fieber & Lutz (1982) do not report using lanthanum chloride in their samples and this might have caused underestimation of the calcium concentrations due to phosphate inteference. These authors found a significant increase in the calcium concentration of the hepatopancreas of *Macrobrachium rosenbergii* during moult stage D_3'''. This concentration of calcium was reduced at the time of moulting to a fifth of the premoult concentration. Net loss of calcium was found to occur during the moult, and this would have to be obtained from the environment, or by the consumption of the old carapace. It was noticed in this work that prawns that had just moulted did not generally

(Fig.33) Variation in the haemolymph [Ca] of adult prawns over the moult cycle. Prawns held in freshwater at 27 °C.



feed on anything but their exuvia. This is common behaviour in many crustacean species studied, and appears to be a rapid method for obtaining calcium and other useful organic components that would otherwise be wasted. (Greenaway 1985).

The increase in haemolymph calcium concentration prior to moulting has been reported for many crustacean species (review by Greenaway 1985), and generally this increase is small, although in some species storage in the haemolymph occurs and much higher concentrations are found. Greenaway (1985) comments that the concentration of ionized calcium probably does not change, and the major fluctuations occur with complexed or bound calcium. Since the samples in this work were acid-digested the calcium fluctuations represent the total haemolymph calcium concentration.

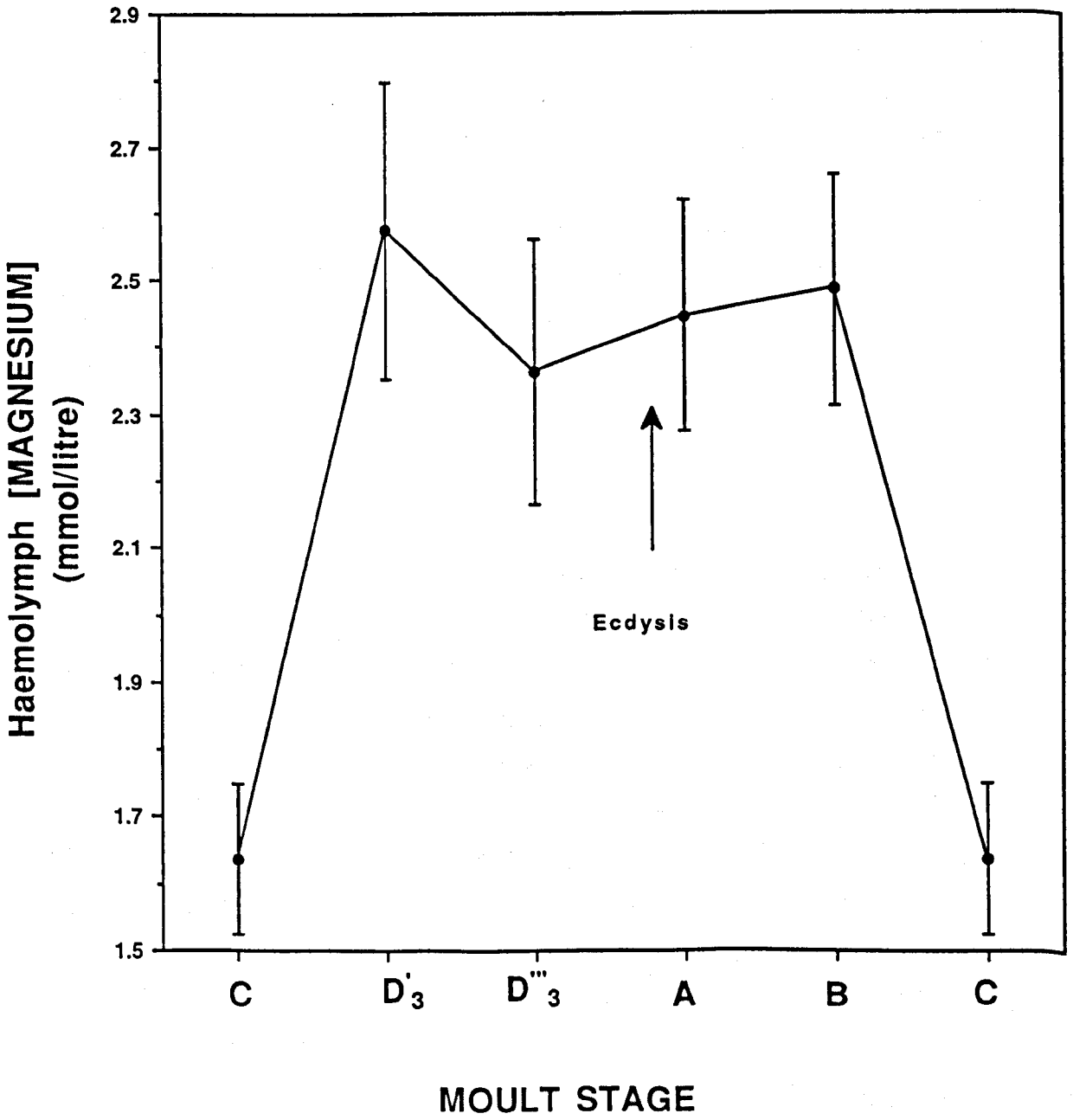
The decrease in calcium concentration that occurs during moult stages A and B has been reported for other species (review by Greenaway 1985), but the timing of these reductions appears to vary according to the time that cuticle calcification begins, and the extent to which the haemolymph is diluted during moulting.

6.3.2.3 Magnesium (Fig.34)

The haemolymph magnesium concentration increased sharply from an intermoult value of 1.636 $mmol.dm^{-3}$ to a concentration of approximately 2.4 $mmol.dm^{-3}$ (significant difference, two sample t-test, $p<0.05$). This concentration was maintained throughout the premoult and postmoult period. There was no significant difference between any of the moult stages D_3'', D_3''', A and B (two sample t-test, $p<0.05$).

The regulation of magnesium over the moult cycle does not agree with the findings of Fieber & Lutz (1985), who found an increase in concentration up to premoult, followed by a gradual decline to an intermoult minimum. The hypothesis of Fieber & Lutz (1985) that magnesium plays some role in the moulting process is not contradicted by these data. Indeed, if the physiological processes of moulting require magnesium it might be expected that maintenance of a constant haemolymph magnesium concentration over the moulting period might be desirable. The magnesium concentrations analysed by Fieber &

(Fig.34) Variation in the haemolymph [Mg] of adult prawns over the moult cycle. Prawns held in freshwater at 27 °C.



Lutz (1985) are very much lower than those found in this work, but there is no satisfactory explanation for this difference. The intermoult magnesium concentration that they measured is much lower than that found by Stern *et al.* (1987), whose results are in agreement with those of the present study.

The ability of *Macrobrachium rosenbergii* to maintain a constant haemolymph magnesium concentration during the moult period suggests a high degree of regulation. This prawn experiences quite wide variations in the haemolymph concentrations of strontium, calcium and copper during this period. The increase in magnesium concentration might explain the seemingly higher oxygen saturation of the haemolymph at the time of the moult. Magnesium is known to affect cooperativity of haemocyanin (section 8.1) and thus the elevated haemolymph magnesium concentration might be increasing the oxygen affinity of the haemocyanin. This would be advantageous since the metabolic changes that occur during the moult are likely to require a greater supply of oxygen to the tissues.

6.3.2.4 Strontium (Fig.35).

The graph showing fluctuations in the haemolymph strontium concentration during the moult cycle reveals a pattern unlike that of calcium or magnesium. There was a steep rise from an intermoult level of 0.030 $mmol.dm^{-3}$ to a peak at stage D_3''' at a concentration of 0.0497 $mmol.dm^{-3}$. This represents an increase in concentration of approximately 65%, and is equivalent to the concentration found in 20°/ooS seawater. The increase in haemolymph strontium concentrations are significant (two sample t-test, $p<0.05$) between moult stages C to D'_3, D'_3 to D'''_3. After stage D'''_3 the haemolymph strontium concentration decreased sharply back towards the intermoult concentrations. There was a significant reduction in the haemolymph strontium concentration between stages D'''_3 to A, and A to B. There was no significant difference between stages B and C.

This increase in strontium concentration during premoult is significant as it does not follow the regulation lines of any of the other ions analysed. The large increase in strontium concentration is unlike the regulation patterns of the other divalent cations (Figs.33,34). The increase in strontium concentration cannot be attributed to a

concentration of the haemolymph for two reasons. Firstly, the haemolymph has the lowest water content at stage D_3' not D_3'''. Secondly, the increase in concentration of 65% is far greater than the percentage changes of any of the other ions. This implies that the strontium concentration is being deliberately raised for some purpose.

The increase in strontium concentration prior to moulting could be explained in terms of the mobilisation of strontium bound in the carapace. Indeed, this would seem to be the most likely source of the ion. This is because the prawns cease to feed several days prior to moulting. Whilst the rise in strontium concentration might be attributed to mobilisation of strontium, the maintenance of this concentration in the haemolymph would require considerable regulatory effort. If the ion were unnecessary it might be expected that a rise might occur, but the loss of that ion by diffusion would follow, especially because the apparent permeability of the prawn is increased around the time of moulting. The conservation of this ion against such a gradient could be explained by a higher affinity for strontium than for the other divalent cations by the regulatory mechanisms. This would have the effect of conserving strontium preferentially to calcium and magnesium in the reabsorption processes of the antennal gland. In this way strontium might be retained as it is mobilised from the carapace.

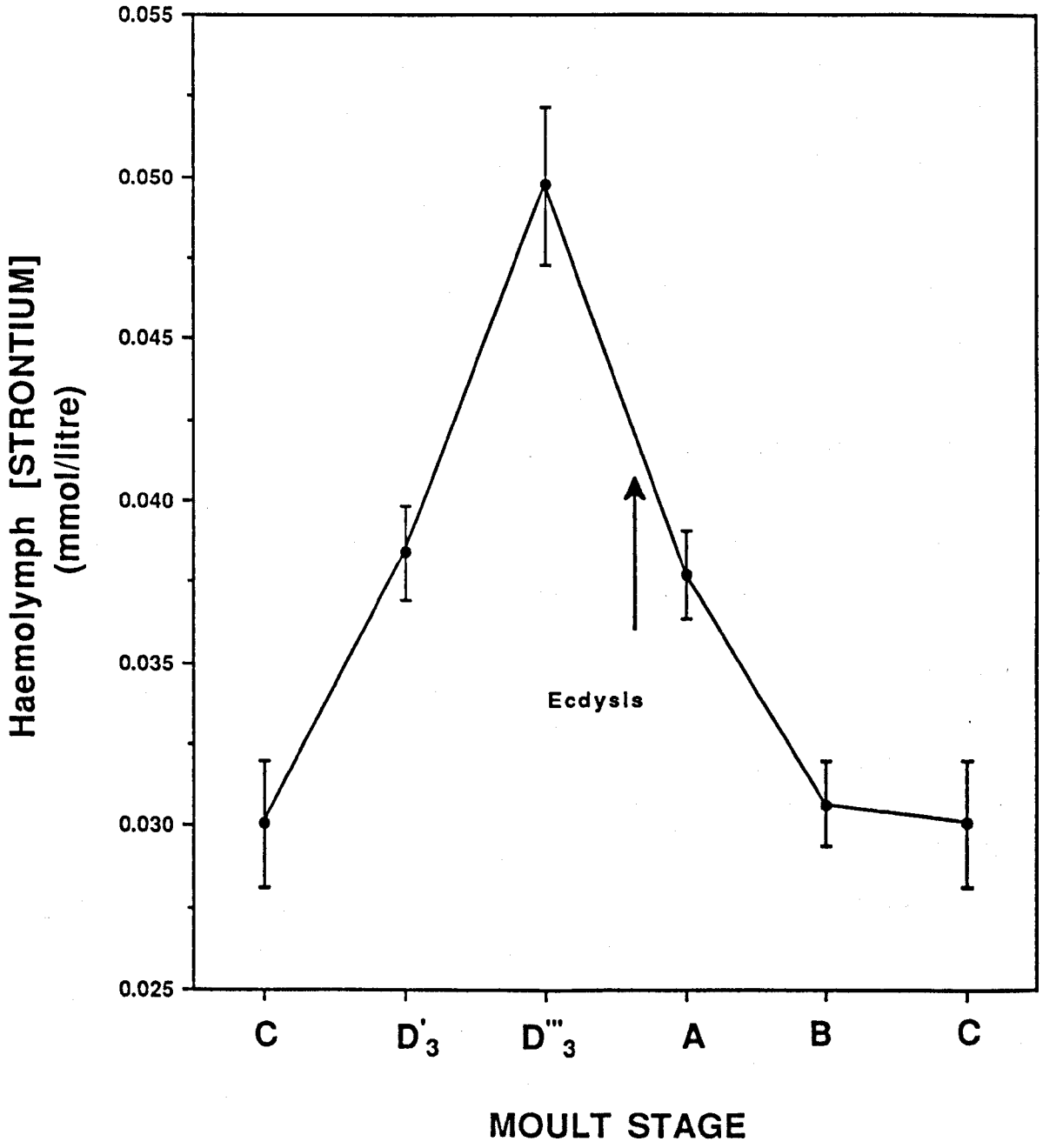
It is interesting to note that the stage at which the strontium concentration is highest is that which immediately precedes moulting. Prawns always moulted within 12-36 hours of being sampled when at this stage. Sampling the prawns did not appear to interfere with the moulting process. This implies that either the haemolymph sample removed was too little to cause an effect, or that the moulting factors were replaced quickly enough to allow moulting to occur.

The rapid rise in the strontium concentration just prior to moulting in *Macrobrachium rosenbergii* agrees with the conclusion made by Bidwell *et al.* (1990) that strontium affects a specific biochemical pathway in the calcification process.

6.3.2.5 Strontium and magnesium (Fig.36)

The double Y-plot of strontium and magnesium regulation reveals that the

(Fig.35) Variation in the haemolymph [Sr] of adult prawns over the moult cycle. Prawns held in freshwater at 27 °C.



two ions are regulated in very different ways. These graphs show that whilst magnesium is maintained at a constantly high concentration during premoult and postmoult, the concentration of strontium increases until just before the moult, whereupon it decreases sharply.

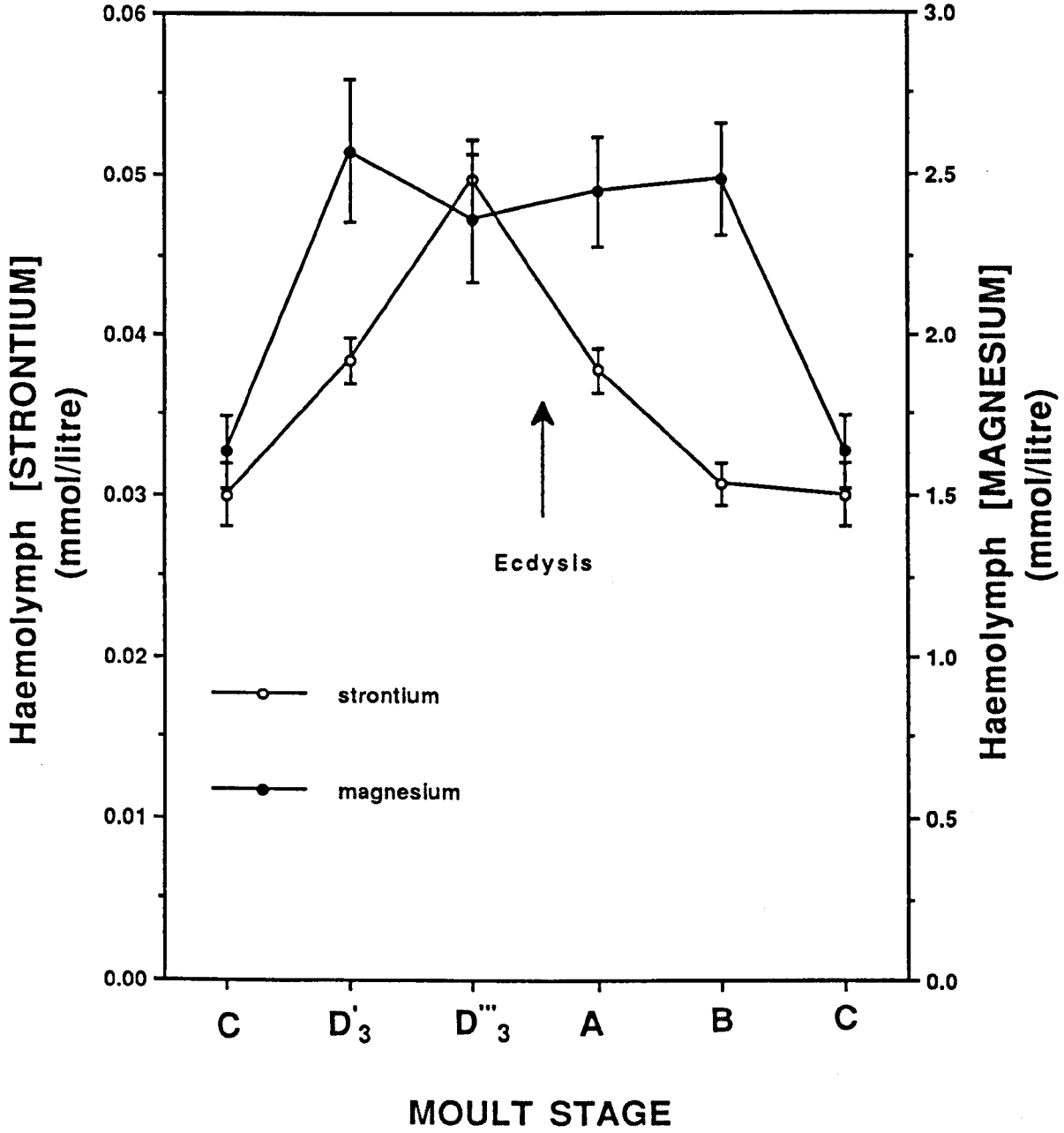
This graph reveals that the strontium regulation mechanism is not directly linked to the regulation of magnesium, as was suggested in section 6.2.2.10. This does not mean that they do not have similar mechanisms for regulation, merely that *Macrobrachium rosenbergii* is capable of regulating the two ions independently. Alternatively, the regulation of these ions are linked but this ceases during the pre- and postmoult stages. This linking and unlinking of ionic regulation has been suggested by Zanders (1980,1981) and Mangum (1983).

The ratios between the two ions vary between the different stages of the moult cycle, although magnesium is held at a stable concentration between stages D_3' and B. The ratio between the strontium concentration at stage D_3''' and the magnesium concentration between stages D_3' and B varies between 47.6 and 51.8. This is very close to the ratio found between strontium and magnesium in the salinity exposure experiment (51.5 $\pm$ 1.031, range 48.5-54.5). Thus, whilst strontium and magnesium seem to be regulated in different ways there still appears to be an underlying relationship.

The question that arises from this work is whether the regulation of strontium dictates the regulation of magnesium, or that the reverse is the case. Since the animals used in this experiments were maintained in freshwater the hypothesis that strontium is dictating magnesium regulation is preferred. The extremely low strontium concentration of the medium means that the uptake of strontium must be quite effective. The closeness of the ratio between strontium and magnesium at moult stage D_3''' and the ratio found between the two in prawns held at different salinities seems more than coincidental. Equally important is the closeness of the ratio between the two ions in prawns maintained in freshwater. Thus, it would seem that the argument is in favour of the concept that magnesium regulation is following that of strontium.

The hypothesis of Fieber & Lutz (1985) that magnesium was required at higher concentration for the correct functioning of the moulting processes due to the

(Fig.36) The variation in the haemolymph [Mg] and [Sr] of adult prawns over the moult cycle. Prawns held in freshwater at 27 °C.



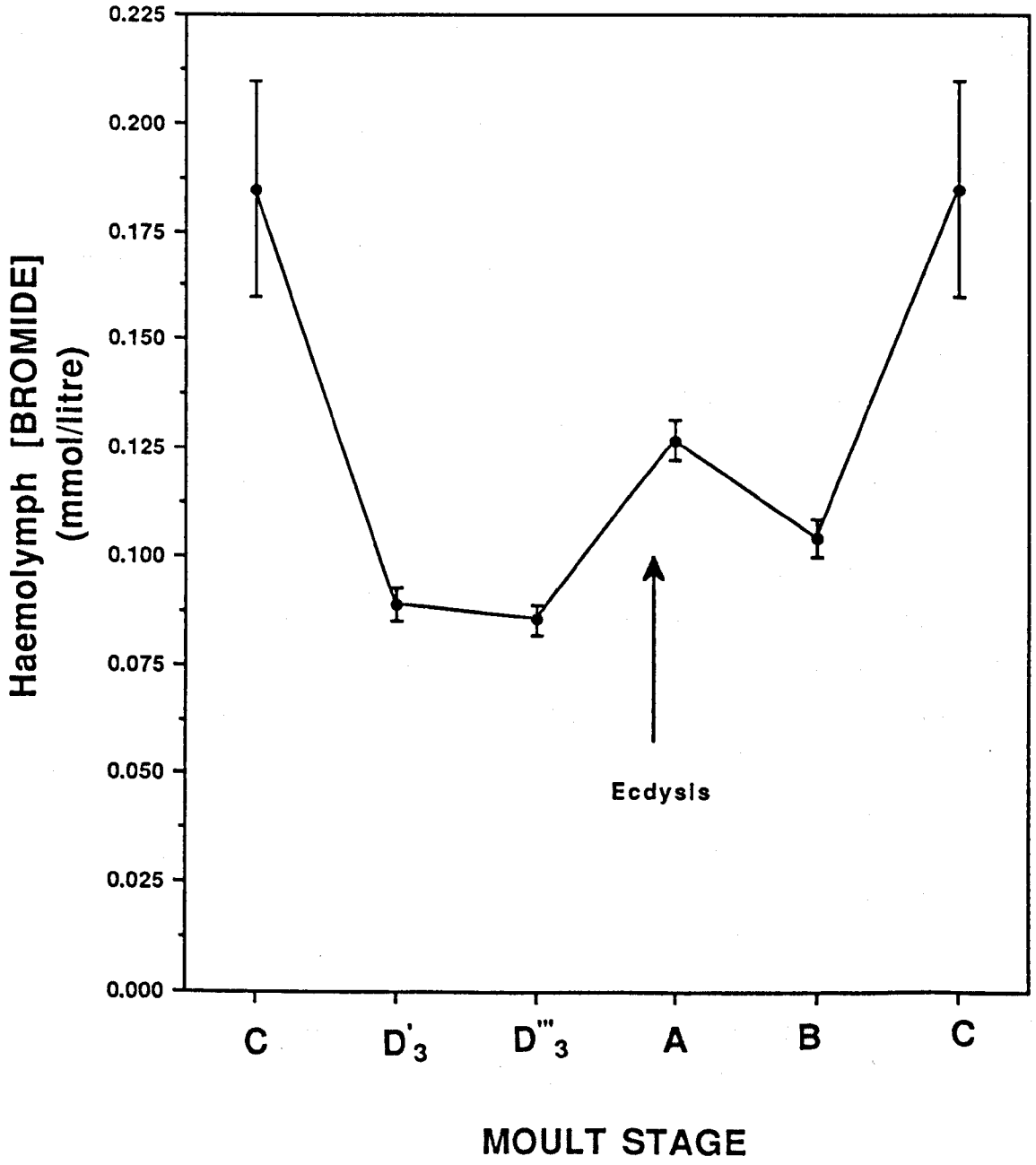
marine ancestry of the prawns can equally be applied to strontium. Indeed, it is even more applicable, as the increase in magnesium concentration is from 1.636 to 2.57 mmol.dm^{-3}. These are equivalent to the magnesium concentrations found in 1.05 and 1.65^{o}/ooS seawater respectively. This is in sharp contrast to strontium which rises from 0.03006 to 0.04973 mmol.dm^{-3}, equivalent to 12.0 and 19.9 ^{o}/ooS seawater respectively. The concept of seawater-type conditions being required does not appear valid with respect to magnesium concentration, but may be in the case of strontium.

6.3.2.6 Bromine (Fig.37)

The haemolymph bromide concentrations were reduced during the premoult and postmoult periods from the intermoult concentration of 0.185 mmol.dm^{-3} to a concentration between 0.0888-0.0855 mmol.dm^{-3}. This represented a reduction in the haemolymph bromide concentration of 54%. The haemolymph bromide concentration increased after moulting (stage A) to a concentration of 0.127 mmol.dm^{-3}, an increase of approximately 33% of the premoult concentration. This was then reduced in stage B to 0.104 mmol.dm^{-3}. The bromide concentration at stage A was 31% less than the intermoult concentration.

The results presented above do not provide a clear indication of what might be occurring to the regulation of bromide during the moult cycle. This is due to the higher concentration of bromide found at the time of moulting relative to the premoult concentration and stage B concentration. It is assumed that the permeability of a crustacean increases at the time of moulting, and this coincides with an increase in the water content of the haemolymph. The result of this is thought to be a loss of ions due to increased permeability, coupled with a dilution of the haemolymph ionic concentration due to the increased water content. This means that ionic concentrations should be maximal prior to moulting, and reduced or minimal at stage A. This was found to be the case for copper, calcium and strontium in this work. The occurrence of an elevated haemolymph bromide concentration in stage A might indicate increased sodium and chloride transport due to the increased permeability of the prawn, although bromide does not appear to be preferentially absorbed by this species (section 6.2.2.6). Another possibility is that the bromide is required for the sclerotisation process which commences immediately postmoult. The requirement for bromine/bromide in the sclerotisation process is

(Fig.37) Fluctuation in the haemolymph [Br] of adult prawns over the moult cycle. Prawns held in freshwater at 27 °C.



plausible since it is found in the scleroproteins of marine invertebrates (section 5.2). If bromine/bromide is required for scleroprotein formation, the haemolymph would be used for bromine/bromide transport, in the same way that tyrosine, and tyrosine derivatives (catechols) are transported to the cuticle. Indeed it is possible that some or all of the bromine/bromide is already part of the tyrosine, catechol molecules. This seems unlikely as bromotyrosines have not been identified in the haemolymph. They are found in scleroprotein, and thus their incorporation probably occurs as the scleroprotein is formed. In seawater, the bromine could be derived from the medium, however, this argument cannot readily be applied to the freshwater medium in which these prawns were maintained. Thus the plausibility of whether the bromide is being incorporated into the cuticle depends upon whether it is found in the cuticle of the prawn when it is found in freshwater. Two samples of the exuviae of larval prawns were found to contain between 14.5-14.9 $mmol.kg^{-1}$, dry weight, (1159-1194 ppm) bromine/bromide.

The high haemolymph concentration of bromide during intermoult might be due to the reduced permeability of the prawn allowing the maintenance of a higher bromine/bromide concentration. Alternatively, the reduction of bromine/bromide during premoult might be due to its removal into the new cuticle, developing beneath the old one. This supposes that bromine incorporation into the carapace is deliberate, and occurs prior to full sclerotisation. It also suggests that bromine is being incorporated into the cuticle structure as a precursor to some latter process e.g. sclerotisation. Another possibility is that the bromine is not required for a particular physiological purpose, but its presence in the cuticle confers some desirable physical property e.g. elasticity, or altered surface energy (sections 5.2,7.2.1).

The high intermoult bromine/bromide concentration might be explained by the fact that analysis of the intermoult haemolymph occurred at SURRC, whilst the other samples were analysed at Imperial College Reactor Centre. This is the least favoured explanation, since the same volumes of the same standard solutions were used for both analyses, and therefore the sample analyses were relative to the same bromide concentration. However, the possibility of a discrepancy must be considered.

6.4 Conclusion

The results of this work show that *Macrobrachium rosenbergii* seems to have a considerable ability to control its internal ionic environment. this control seems to be maintained even during the pre- and postmoult stages of the moult cycle. Whether this is because the prawn does not become extremely permeable at this stage, or there is increased ionic reabsorption is not clear. This strong osmoregulatory ability in the adult prawn might only become functional after metamorphosis as suggested by Pannikar (1941). If this were the case, the larval stages might be confined to brackishwater by their inability to survive the osmotic stress exerted by the freshwater environment.

Read's (1984) hypothesis that a difference in permeability between the stage I and stage II larvae of *Macrobrachium petersi* allowed the stage I larva to survive in freshwater suggests a possible difference in the composition of their cuticles. If the key to the stage I larva's ability to tolerate freshwater is related to the composition of its cuticle, this might change at metamorphosis, allowing the postlarvae to tolerate freshwater. This change of composition might take the form of a different ratio of sclerotised to calcified cuticle, or just the extent of calcification and sclerotisation. Bromide and strontium are both found in cuticles of crustaceans, but whether they have active roles in sclerotisation and calcification is unknown. These questions are considered in the next section.

CHAPTER 7 - Larval and postlarval calcification.

7.1. Structure and composition of the cuticle.

The arthropod integument is a composite structure and can have a variety of physical properties depending upon the different levels of inclusion of its constituents and the manner in which they interact with one another. The literature concerning the structure of the arthropodan integument has been extensively reviewed (Richards 1951; Dennell 1960; Hackman 1971; Neville 1975; Stevenson 1985). Those studies concerning the structure of the crustacean cuticle have concentrated almost entirely upon the mineralisation process, far more so than in insects. Conversely, studies of insect cuticles have covered areas largely ignored in the work on crustaceans such as, sclerotisation, cuticle lipids and proteins (Dennell 1960, Stevenson 1985). This perhaps stresses the structural differences between the arthropod classes and not similarities.

The general appearance of the crustacean integument is that of a series of layers; these have been divided according to differences in composition or function. The principal layers according to Richards (1951) are:

A) Epicuticle
B) Procuticle
    i) Pre-ecdysial procuticle
    ii) Post-ecdysial procuticle
        a) Principal layer
        b) Membranous layer
C) Epidermis
D) Basement membrane

Epicuticle

This is the thin outer layer of the cuticle and is distinguished from the other layers by the absence of chitin. It is principally composed of protein, lipid and calcium salts. Much of the protein in the epicuticle is sclerotised (quinone tanned). The external surface of the epicuticle has a thin surface membrane that resists dissolution in concentrated HCl. This has been shown to consist of straight chained paraffins (Dennell 1960).

Procuticle

This is divided into two layers, the difference between the two is their time of formation. The pre-ecdysial layer is secreted before, and the post-ecdysial layer after ecdysis. The post-ecdysial layer is further divided into principal and membranous layers. The distinction here is that the membranous layer does not contain calcium. The rest of the procuticle layers contain primarily chitin, protein and calcium salts.

Pre-ecdysial procuticle may be lightly tanned (sclerotised) and resists digestion by moulting fluid during premoult. Its principal role may be that of protection of the new cuticle whilst the old cuticle is being resorbed. In some crustaceans, this layer can be further subdivided into the outer, pigmented layer, and the inner non-pigmented layer (Dall 1965). The pigmented layer gains its colouration from sclerotin or melanoproteins (Hackman 1964).

Post-ecdysial procuticle is not tanned significantly and is calcified, the calcium crystals (as calcite) parallel the chitin microfibrils and in general, the more calcium present, the less protein (Neville 1975).

The appearance of the chitin in the procuticle is that of a lamellar structure. This has been suggested to be due to the way in which the chitin microfibrils orient themselves as they are formed. It is thought that the fibrils are arranged helicoidally, giving a lamellar appearance when viewed in section (Stevenson 1985).

Epidermis

This is a single layer of cells that secretes the epicuticle, procuticle, and moulting fluid. Pigment cells and connective tissue cells may be mixed with epidermal cells, and some may be differentiated into sensory cells. The cells of the epidermis grow during premoult and regress during postmoult.

Tegumental glands and pore canals

The crustacean integument also contains numerous pores and glands. The role of the pore canals is thought to be associated with either transport of substances to the surface of the cuticle, or to aid the deposition of substances within the cuticle.

Table (XXIX) The various products and functions of crustacean tegumental glands - adapted from Alexander (1989).

Species	Location	Function	Reference
Talorchestia martensii	Cephalic	Lubrication,food entanglement,digestion	a
Ligia exotica	Oesophagus	Ingestion, digestion	b
Alpheus edwardsii	Labrum Labium (Paragnath)	Lubrication	c
Palaemon serratus	Paragnaths	Food entanglement, ingestion	d
Metapenaeus mastersii	Integument	Lubrication	e
Homarus vulgaris	Statocyst	Statolith attachment	f
Homarus americanus	Pleopod	Egg attachment	g
Gammarus pulex	Cephalic	Dopamine,catecholamine production	h
Palaemonetes pugio	Gills	Osmoregulation,phenol oxidase secretion	i
Orconectes propinquus	Pleopods, third maxillipeds	Tyrosinase (phenol oxidase) secretion	j
Homarus americanus	Pleopod	Cuticle and egg coat hardening	k
Homarus americanus	Eyestalk	Associated with moulting	l
Carcinus maenas	Periopod merus	Phenolic tanning and pigmentation of cuticle	g

a) Shyamasundari (1979)
b) Kumari *et al.* (1983)
c) Rajendranath *et al.* (1984)
d) Alexander (1989)
e) Dall (1965)
f) Lange & Yonge (1935)
g) Krishnan (1951)
h) Eloffson *et al.* (1978)
i) Doughtie & Rao (1982)
j) Stevenson & Schneider (1962)
k) Johnson & Talbot (1987)
l) Arsenault *et al.* (1979)

Tegumental glands have also been implicated in improving the efficiency of gaseous exchange in the gills of the shore crab (Dennell 1960). Tegumental glands are also found underlying the crustacean integument, these are connected to the external surface of the integument by canals, their role is also uncertain. Yonge (1932) suggested that the tegumental glands might secrete the epicuticle, but this was rejected subsequently due to the irregular distribution of the glands. The secretions of the tegumental glands have been studied by a number of authors and more than one function has been ascribed to them (Table XXIX). Doughtie & Rao (1982) found there to be two types of gland. They found one type to be of a secretory form containing secretory vesicles (Type A) and the other seemed to have an osmoregulatory function (Type B). The type B tegumental gland appears to be innervated. The activity of the two glands increased soon after ecdysis and it was concluded that they were involved with phenol oxidase secretion and osmoregulation.

7.2 Cuticle hardening - sclerotisation and calcification.

The protein content of crustacean cuticles varies between species and according to their mode of life. The Decapoda have replaced much of their cuticular protein with calcium salts, and this can be seen in their high ash values. However, the horseshoe crab, *Limulus polyphemus* (Merostomata) whilst unrelated to the Crustacea, is a marine arthropod. It has a very low ash value (0.095% by weight, Richards 1956), its composition being closer to that of the spiders, and its cuticle contains only a small proportion of salts, which are mainly present as phosphates. The cuticle composition of the Merostomata is believed to resemble that of ancestral Crustacea (Vinogradov 1953).

7.2.1 Sclerotisation

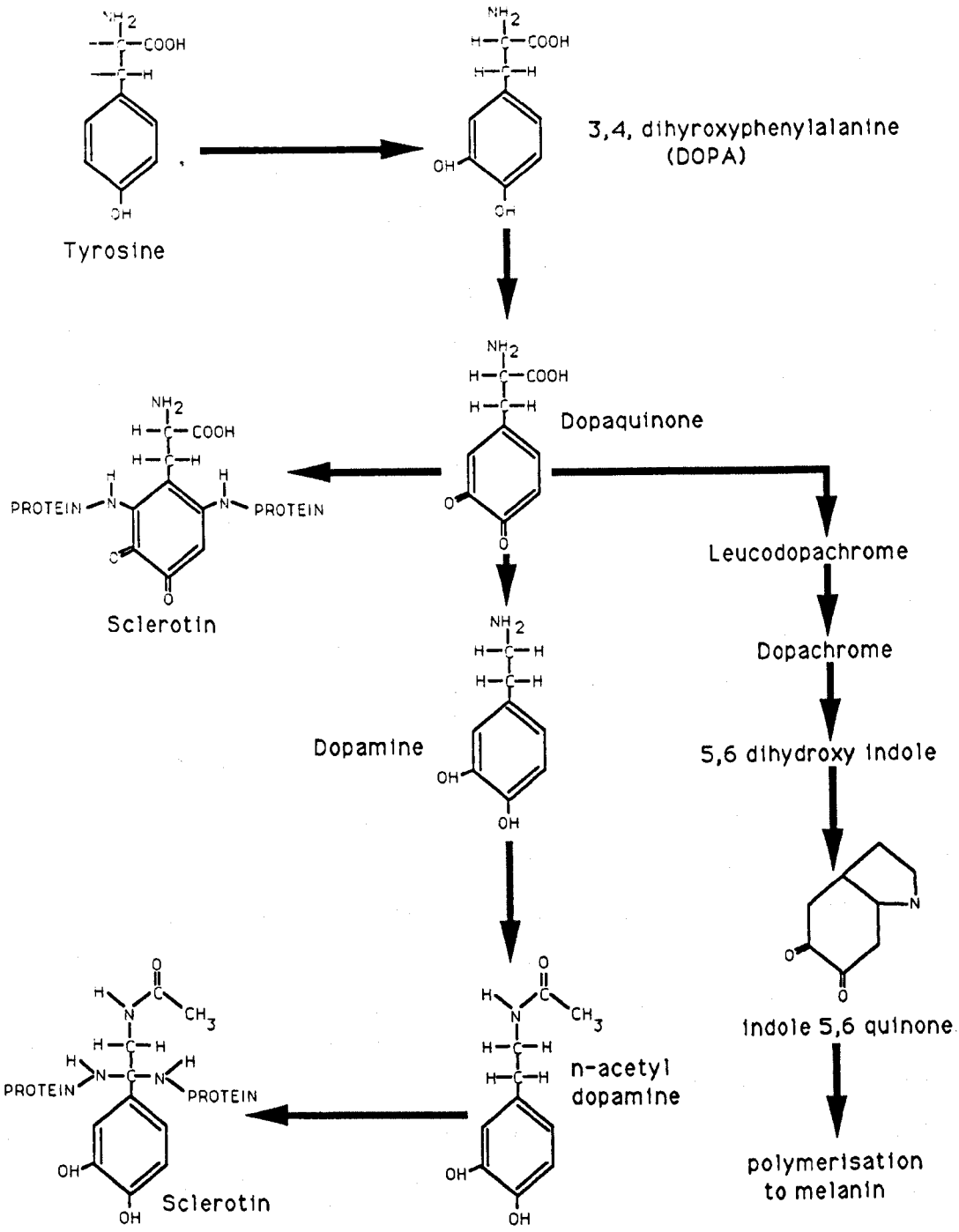
The different proteins present in arthropod cuticles can be arbitrarily divided into types depending upon the nature of their bonding within the cuticle. The protein that is the most difficult to remove is generally considered to be sclerotised (covalently bound). Although detailed studies of sclerotisation have been made on the puparia of flies and the ootheca of the cockroach, enough information is available to show

that similar systems occur in all other arthropods investigated. In crustaceans, sclerotisation has been shown to occur in a number of genera, including *Cancer, Carcinus, Gammarus, Homarus,* and *Orconectes* (Hackman 1971).

The degree to which cuticular protein is sclerotised depends upon the mode of life of the organism and the location in the cuticle of the protein. Sclerotised protein is found most abundantly in cuticle areas that are subject to abrasion (Dennell 1960). It is the occurrence of large numbers of tegumental glands with heavily sclerotised cuticle that suggests the link between the tegumental glands and sclerotisation of protein. Calcification of the cuticle progresses from the epicuticle inward. The colourm of sclerotised cuticles varies between a light amber to dark black. The intensity of the colour is an indication of the extent of sclerotisation (Dennell 1960).

The process of sclerotisation involves the conversion of o-diphenols into their corresponding quinones by a phenol oxidase (Fig.38). The quinone groups then react with certain side groups on adjacent proteins linking them together. The o-diphenols have their origin in extracellular tyrosine. This is oxidised to DOPA (3,4-dihydroxyphenylalanine) and then to dopaquinone. The dopaquinone either reacts with amino groups, sulfhydryl groups, and the eta-alanine groups of lysine on adjacent proteins to form strutures crosslinked with covalent bonds (Hackman 1971). Alternatively, dopaquinone could be decarboxylated and acetylated to give *N*-acetyldopamine (Andersen 1976). The *N*-acetyldopamine then reacts with amino groups on adjacent proteins to link them. The haemolymph concentration of tyrosine increases prior to ecdysis in *Calliophora* (Spearman 1973) the implication being that this is the source of the tyrosine for sclerotisation. Since crustaceans are less sclerotised, this increase in blood tyrosine concentration may be less easy to detect. In the absence of free phenolic substances, tyrosine attached to the protein chains may possibly be oxidised to quinones (L'Helias 1970).

Dopaquinone can be converted to indole-5,6-quinone which then undergoes polymerisation to form melanin (Spearman 1973). The processes of sclerotisation and formation of black pigments of the melanin type, and the repair of wounds, although quite separate, are related (Hackmann 1971). The relationship between these processes is in their use of the same precursors - aromatic amines and quinones (DOPA, dopaquinone). The enzymes systems that activate the metabolic pathways to the different reaction



(Fig. 38) The relationship between Tyrosine metabolism and the processes of sclerotisation and melanisation.

products are similar, although the method of activation appears to be different. It is the activation of these processes that causes them to occur at specific times, or in relation to specific events.

Hackman (1971) cites various evidence in support of the theory that sclerotisation and melanisation are separate processes. This work reveals that, in albino insects, the sclerotisation process occurs normally as in pigmented wild types. Other evidence from heavily pigmented mutants shows that the enzyme activity is affected by a component in the larval cuticle, and is not related to the type of enzyme, and that sclerotisation occurs similarly in both mutant and wild type. This suggests that local melanin synthesis occurs in the cuticle, and that the precursor required to activate the proenzyme is already in place in the cuticle (Hackman & Goldberg 1968).

It should be noted that melanin has not been found in the two caridean prawns *Crangon crangon*, and *Palaemon serratus* (Goodwin 1960). It seems that these prawns contain ommochromes which can also appear black. This might be significant with respect to *Macrobrachium rosenbergii* as it too is a member of the family Palaemonidae.

Bromine (and iodine) is found in the scleroproteins of marine molluscs, sponges, annelids and crustaceans (section 5.2, 5.3). The reason for its presence in these scleroproteins is uncertain. It has been postulated that it is present adventitiously due to the oxidising reactions involved in sclerotisation, oxidising bromide to free bromine. This subsequently reacts with tyrosyl groups in proteins that are part of the sclerotisation process. What is not clear is whether the bromine is present only in the tyrosine molecules that are part of the proteins, or whether it is combined in those groups that have formed crosslinks in the sclerotisation reaction. It may be that the presence of bromine in these groups modifies the physical properties of the scleroprotein in some way.

In insects, sclerotisation occurs shortly after the moult, starting from the outer layers and proceeding inwards. In a crustacean, this might confer some structural strength whilst the slower process of calcification occurs. The degree of sclerotisation in the final composition of the adult crustacean cuticle does not appear to be as great as in that of the insects (Stevenson 1985). This is unsurprising considering the degree of mineralisation in the crustaceans. The sclerotised cuticle is present in the Merostomata,

insects and chelicerates, and is probably present to some degree in all crustaceans. What is of interest is whether or not the calcified cuticle of the modern Crustacea replaced a predominantly sclerotised, unmineralised ancestral cuticle. If this occurred then was there an advantage in this process? The phylum Arthropoda is now considered to be a polyphyletic collection which should consist of: phylum Crustacea, Chelicerata, and Arthropoda (Manton & Anderson 1979). This may explain why the Crustacea developed a calcereous cuticle and the other phyla did not (i.e. there are no particularly calcified insects or chelicerates).

7.2.2 Calcification

The primary method by which crustaceans harden their cuticle is calcification, whereby calcium salts are deposited within the chitin-protein matrix of the cuticle. The principle calcium salts found in the calcified cuticle are $CaCO_3$ and $Ca_3(PO_4)_2$ (Richards 1951) and are found as calcite, vaterite and hydroxyapatite (Neville 1975). Vinogradov (1953) lists aragonite as a possible form of $CaCO_3$ found in hard structures in invertebrates. Magnesium is the second most prevalent element in the cuticle and other minor elements include strontium, barium, silicon and fluorine (Vinogradov 1953; Dodd 1967; Gibbs & Bryan 1972, Brannon & Rao 1979; Wickins 1984). The presence of these minor ions is considered by Dodd (1967) who states:

> *"The tacit assumption is commonly made that minor and trace cations are present as substitutions for Calcium in the calcite or aragonite crystal lattice. The circumstantial evidence for this is strong, and in some carbonate skeletons substitution can be proven. Magnesium in many organisms clearly substitutes for calcium in the calcite lattice, as is proven by the alteration of lattice dimensions which are dependent on the Ca:Mg ratio."*

Magnesium is always more abundant in chemically precipitated calcite ($CaCO_3$) than in aragonite ($CaCO_3$). The explanation for this is that the small ionic radius of magnesium allows it to substitute for calcium more readily in calcite, which is isostructural with magnesite ($MgCO_3$), than in aragonite. Strontium on the other hand is commonly (though not invariably) more abundant in aragonite which has a crystal lattice

isostructural with strontianite ($SrCO_3$). Dodd (1967) continues by suggesting that trace and minor elements cannot be proven to be substituted into the lattice where their concentration is low. It is suggested that in molluscan shells some of the trace elements are present as particulate inclusions present at the time of shell deposition. Another explanation is that the porous nature of the shell allows the inclusion of seawater as a liquid phase, and upon drying and analysis, the trace elements present would be counted as part of the skeletal composition. This is possibly more important with regard to the composition of calcified structures in marine organisms than freshwater organisms since the level of trace elements is lower in freshwater. The cuticle of crustaceans is by no means a dry inert structure, the organic component is extensively cycled and has a high water component (Stevenson 1985). The cuticle of intermoult *Macrobrachium rosenbergii* contains approximately 42% water (present study). If this water is incorporated from the body fluids its trace element composition could be expected to reflect the haemolymph composition to some extent. However, if this water is derived from the medium it should resemble the ionic composition of the medium. It is possible that some trace elements are adsorbed onto the cuticle, although this is likely to be significant only for those elements present at extremely low concentration (e.g. uranium and radium, Dodd 1967).

The ratios of magnesium, calcium, strontium and barium in calcified structures have been used to estimate the ionic ratios of the external medium and its temperature in the study of evolutionary relationships between animals, paleoclimatology and history of the oceans (Dodd 1967). The concept being that the ratios of the elements in the various crystal structures changes depend upon the ambient temperature and their environmental concentration. However, it seems from Dodd's (1967) review that only the relationship between calcium and the other elements has been considered. This is presumably due to the concept that the other elements are incorporated accidentally instead of calcium.

The use of ionic ratios in calcified tissue as a means of determining environmental parameters can only be an approximate method for two reasons. Firstly, analysis of fossil material does not always reveal exact compositions of skeletons due to post depositional changes in composition (diagenesis), although careful selection of material can avoid this (Dodd 1967). Secondly, the the components for calcification are

derived from body fluids permeating the organic lattice, so a change in environmental composition could be compensated for by increased uptake or excretion by the animal, thereby maintaining its ionic composition. A changing skeletal ratio would suggest a modification of blood composition, this could then be suggested to be due to a changing environmental concentration. However, this would take place only in evolutionary time. Most of the compositional changes of hard structures have been studied with respect to geological time by analysing fossilised skeletons. These are separated from each other by very long periods of time, this allows for possible modifications in tissue fluid composition due to changing environmental ratios. This concept relies on the assumption that the elements incorporated into the skeletal structures have a principle function as structural components. This might be so for calcium and magnesium, although both have roles in the functioning of enzymes (Fieber & Lutz 1985). Strontium does not play a significant role in calcification and yet the ratio of calcium to strontium has been used as an indicator of the composition of ancient seawater and climates. This is because a relationship has been found between the two in the calcified tissues of living animals (Dodd 1967). This would be acceptable provided that strontium does not have a specific physiological role. If it did, then regulation of the ion might compensate for any environmental changes. The deciding factor would be whether the threshold concentration for its physiological role was below that found in the blood, and therefore whether the ion was present in excess of the physiological requirement. This is very likely for calcium, quite likely for magnesium, and unlikely for strontium due to its low abundance. The essentiality of strontium is as yet unproven, although it is considered to be likely to be essential (Underwood 1977). The strontium composition of the carapace of *Macrobrachium rosenbergii* is of interest since it is a freshwater animal with a recent estuarine history. Depending on the concentration in the blood and carapace, this could show whether the seawater concentration of strontium is in excess for this animals' requirements, or even whether it is not required at all. If found in the blood of freshwater animals, this would suggest some sort of role, because considerable concentration from the food or environment would be required (strontium concentration in seawater 8.1 ppm, freshwater 0.08 ppm, Bowen 1966). The presence of strontium in the carapace of the freshwater prawn would suggest that it was incorporated from the body fluids, as environmental concentrations are so low it would be unlikely to be incorporated directly from the medium.

has been studied in relation to a number of environmental factors. Wickins (1984) studied changes in mineralisation in relation to reduced pH in *Penaeus monodon*. He found reduced pH caused elevated magnesium and slightly reduced strontium in the carapace, but concluded that overall the animals regulated the concentrations of the three ions well in all treatments. Brannon & Rao (1978) investigated the relationship between calcium, strontium, and barium in the mineralisation of *Palaemonetes pugio* on exposure to elevated environmental levels of barite ($BaCO_3$). They found that barium was incorporated preferentially to strontium, when the two were compared with the calcium concentration in the carapace. This contrasted with the hepatopancreas and abdominal muscle in which both barium and strontium were discriminated for relative to calcium. Interestingly, the strontium levels of the muscle rose from an intermoult value of 100 ppm (dry weight) to 1000 ppm during premoult, but no data on later moult stages were presented. Over the same period calcium decreased very slightly (900 ppm - 800 ppm). Gibbs & Bryan (1972) studied the relationship between the environmental ratio of strontium and magnesium to calcium on carapace mineralisation in *Uca burgersi*, and *Carcinus maenas*. Their conclusions were that the calcium to strontium ratio was a function of the environmental ratio, and the calcium:magnesium ratio was more or less constant.

7.2.3 Phosphates

Amorphous phosphates are known in crustaceans (Vinogradov 1953) and are found in association with $CaCo_3$. In crustaceans the ratio of $CaCo_3$: $MgCO_3$: $Ca_3(PO_4)_2$ is 80:8:12. In the Stomatopoda (mantis shrimps) the ratio of $CaCo_3$: $Ca_3(PO_4)_2$ is 1:1. Under certain environmental conditions involving, temperature, pH, and salinity, $CaHPO_4$ changes readily into $CaHPO_4.2H_2O$, then into $Ca_3P_2O_8$, this may then form apatite. Apatite, a calcium phosphate has a general formula $Ca_3(PO_4)_2.nCaX_2$. The X can be OH (hydroxyapatite), F (fluorapatite), Cl (chlorapatite) (Vinogradov 1953). Br and I can also be present in the apatite X group.

In his conclusion concerning the effect of the salt composition of seawater on the composition of organisms, Vinogradov (1953) considers the calcification of skeletons. He suggests that the undersaturation of $CaCO_3$ in Arctic and abyssal regions of

the oceans, due to the higher partial pressure of CO_2 and low temperatures, prevents the calcification process, or renders it more difficult. He lists as evidence for this, the prevalence of chitinous crustacean plankton, silicious diatoms and radiolarians, and other organisms without $CaCO_3$ in these environments. This is in contrast to the tropical regions to which corals are confined. This seawater is supersaturated with $CaCO_3$ and generally does not experience any freshwater runoff.

Coral reefs abound with calcareous invertebrates of many phyla. The problem of undersaturation of $CaCO_3$ in Arctic and cold water might explain the high fluoride content of the Euphausiids so far studied (section 5.1). The deposition of an apatite, or fluorapatite cuticle would be considerably easier in the low temperature waters that these animals inhabit.

The influence of freshwater on calcification has also been considered by Vinogradov (1953). He states that invertebrates which migrate into brackish and freshwater lose some or all of the mineral part of their skeletons. His evidence is mainly related to changes that occur in those invertebrates with open circulatory systems. The possession of a circulatory system buffered from the environment by the chitinous exoskeleton, allows a more efficient regulation of the internal environment, and hence the maintenance of internal levels of elements against considerable concentration gradients. It might be this that allows *Macrobrachium rosenbergii* and other species to retain their calcereous skeletons in freshwater.

7.3. Conclusion

Within the Decapoda the degree of calcification of the cuticle is linked very closely to the mode of life. The reptantian decapods are heavily calcified and are predominantly benthic in their habitat, natantians are less heavily calcified and are able to swim (Barnes 1980). All of the Decapoda, with the exception of some entirely freshwater species, have planktonic larvae. The degree of calcification of these larvae does not appear to have been reported. An assumption might be that the larvae have an exoskeleton similar to the adults, although less heavily calcified to allow for their

planktonic existence. However, it might be that the larval integument is more closely related to that of an ancestral form that may not have been calcified, or utilised a different form of calcification (e.g. apatite). The composition of the larvae of *Macrobrachium rosenbergii* is unknown, and as such it is not known what changes if any, occur at metamorphosis. It is known that the mode of life changes from planktonic to benthic, but whether this corresponds to a change in composition thereby changing the density of the larvae is unclear. Planktonic forms of other crustacean species lack calcification (Vinogradov 1953) but this may be due to an adaptation to planktonic life, rather than a primitive feature.

The presence of bromine in scleroprotein may change at metamorphosis as the availability of bromide in the freshwater environment is so much lower than the marine environment (freshwater= 0.2 ppm, seawater= 68 ppm, Bowen 1966). If this were found to occur it would suggest that the postlarvae may be using chloride instead of bromide for their scleroproteins as suggested by Hunt (1984) for other freshwater invertebrates. Alternatively, the maintenance of bromide in scleroprotein would imply a direct role, and presumably an endogenous source of bromide. Any differences in bromide concentration between larval and postlarval stages would have to be considered in the light of any changes in the concentrations of calcium, magnesium and strontium, as these are also part of the calcification process.

7.4 Composition of larval and postlarval *Macrobrachium rosenbergii.*

The intention of this experiment was to try and reveal any compositional changes during the development of larval and postlarval *Macrobrachium rosenbergii.* More specifically, to study any changes that may occur in the concentrations of bromine, calcium, magnesium and strontium.

The hope was that the mechanism by which the larval cuticle is hardened might be elucidated. It is known that the adult prawns have a predominantly calcified exoskeleton, and it seems to be assumed that the larval exoskeleton is calcified as well, although perhaps not to the same extent. The larval requirement for bromide in its rearing water (sections 4.6,5.4) suggested some role for bromide, but whether this is involved in

sclerotisation or some physiological process is unknown. Since larval prawns appeared to have moulting/cuticle problems in the absence of bromide, it was decided to investigate the concentration of bromide in the larvae through metamorphosis into postlarvae. If the bromide was implicated in the composition of the larval cuticle, changes in its concentration might be expected to be found between larvae and postlarvae. Similarly, if the emphasis on calcification changed at metamorphosis it might be expected that calcium, magnesium and strontium concentrations changed at this time.

A second consideration was the relationship between strontium, calcium and magnesium in the postlarvae. It has been proposed that the relative concentrations of these ions in the cuticle reflect the interaction between their environmental concentration and the concentration in the body fluids of the crustacean. In this case, the concentrations found in whole bodies of postlarvae should reflect the environmental conditions under which they were maintained i.e. fresh or saline water.

The method for detecting any changes in the degree or method of cuticle hardening was to rear larvae in a known concentration of bromide, calcium, strontium and magnesium past metamorphosis. At metamorphosis the postlarvae would be split into two groups, one group would be transferred to freshwater, the other left in the saline rearing medium. If there was a compositional change between larvae and postlarvae this should be detected irrespective of the salinity of the rearing medium. This is because the stage XI larvae and newly metamorphosed postlarvae are morphologically similar. This being so, there should not be a great change in their relative compositions. If, however, the carapace composition is a reflection of the environmental concentrations of the elements studied, the postlarvae held in the saline rearing medium should have a similar composition to the larvae.

7.4.1 Materials and methods

Larvae were spawned and reared as described in section 3.2. The rearing medium chosen was artificial seawater since it enabled the concentration of the bromine and strontium to be controlled by known addition. Since entire batches of larvae were required to provide sufficient sample material, the larvae used in this experiment came from a series of spawnings. However, the postlarvae used were all from the same spawning

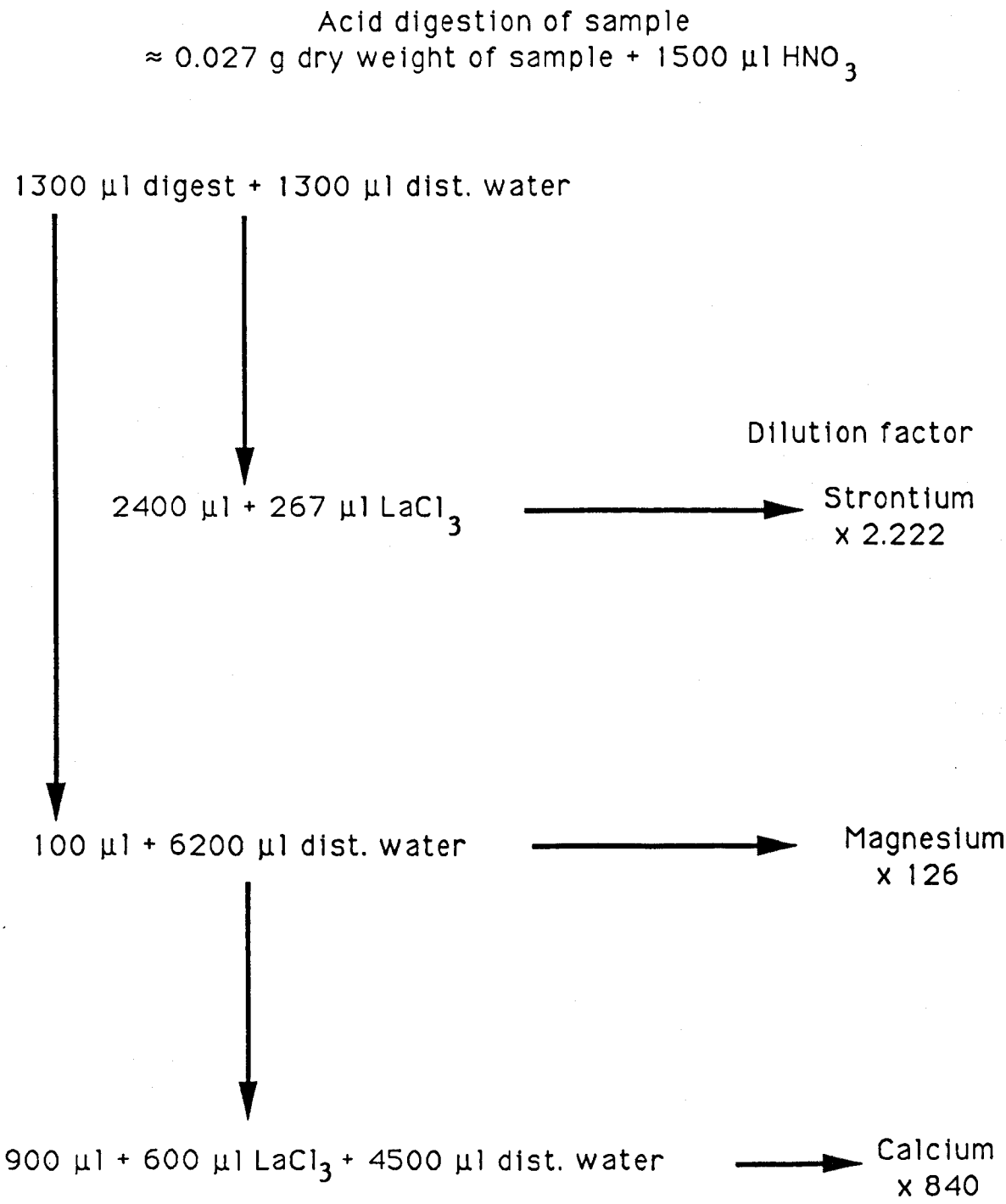
and rearing tank. Newly metamorphosed postlarve were separated into two groups, one maintained in 12 °/oo seawater, the other in recirculated freshwater. The salinity of the seawater rearing media was determined by using a refractometer and maintained at 12 °/ooS. These groups of postlarvae were sampled after 1 and 7 days exposure to their respective media.

Larvae and postlarvae were sampled by filtering the rearing water through a 350μm mesh. This allowed artemia, and small detritus to pass through, leaving the larvae. The prawns were then suspended in deionised water and any large detritus particles or dead larvae were allowed to settle. This took ≈2-3 minutes. The prawns were then decanted back onto the 350 μm mesh, blotted dry and placed in preweighed 'Eppendorf' tubes for wet weight determinations. A similar weight of sample was placed in each tube to ensure that, after dilution, the ionic concentrations of the samples would all be in approximately the same range. After weighing, the tubes were placed in a drying oven at 60°C for 48 hours, after which they were reweighed and stored prior to analysis.

Samples for metal analysis were digested in 'Aristar' 16N nitric acid in the 'Eppendorf' tubes, which were sealed with PTFE tape. This allowed some of the gas evolved from the digestion to escape. To prevent evaporation the samples were placed in a refrigerator during digestion and prior to analysis. Dilution and analysis was performed within two days of digestion. A digestion and dilution protocol for the larval and postlarval samples is given in Fig.39. Analytical methods for the ions studied are described in section 3.3.4.

Larval samples for bromine/bromide NAA were analysed as described in section 3.3.6.2. The approximate dry weight of these samples was 0.02 g. Larval exuviae were collected from the surface of a rearing tank. The exuviae float when they are shed and can be collected on a nylon mesh. The larval exuviae were suspended in deionised water twice to rinse off seawater, and then dried at 60°C for 48 hours. The exuviae were analysed in the same manner as the larvae, although the sample weights were considerably lower (0.0049 g)

(Fig.39) Digestion and dilution protocol for ionic analysis of larvae and postlarvae.



7.4.2 Results and discussion

All graphs were plotted as means ± the standard error of the mean. The quantity of larvae required to provide sufficient material for analysis was considerable. For the early larval stages (I - V) the entire batch of larvae would be required for analysis. This reduced the number of possible replicates to three. The different durations of rearing times for the various stages used inevitably had an effect on the composition of the larvae since evaporation caused slight salinity changes. The possibility of depletion of strontium and bromide in the rearing water due to uptake by the larvae was not quantified.

7.4.2.1 Water content (Fig.40)

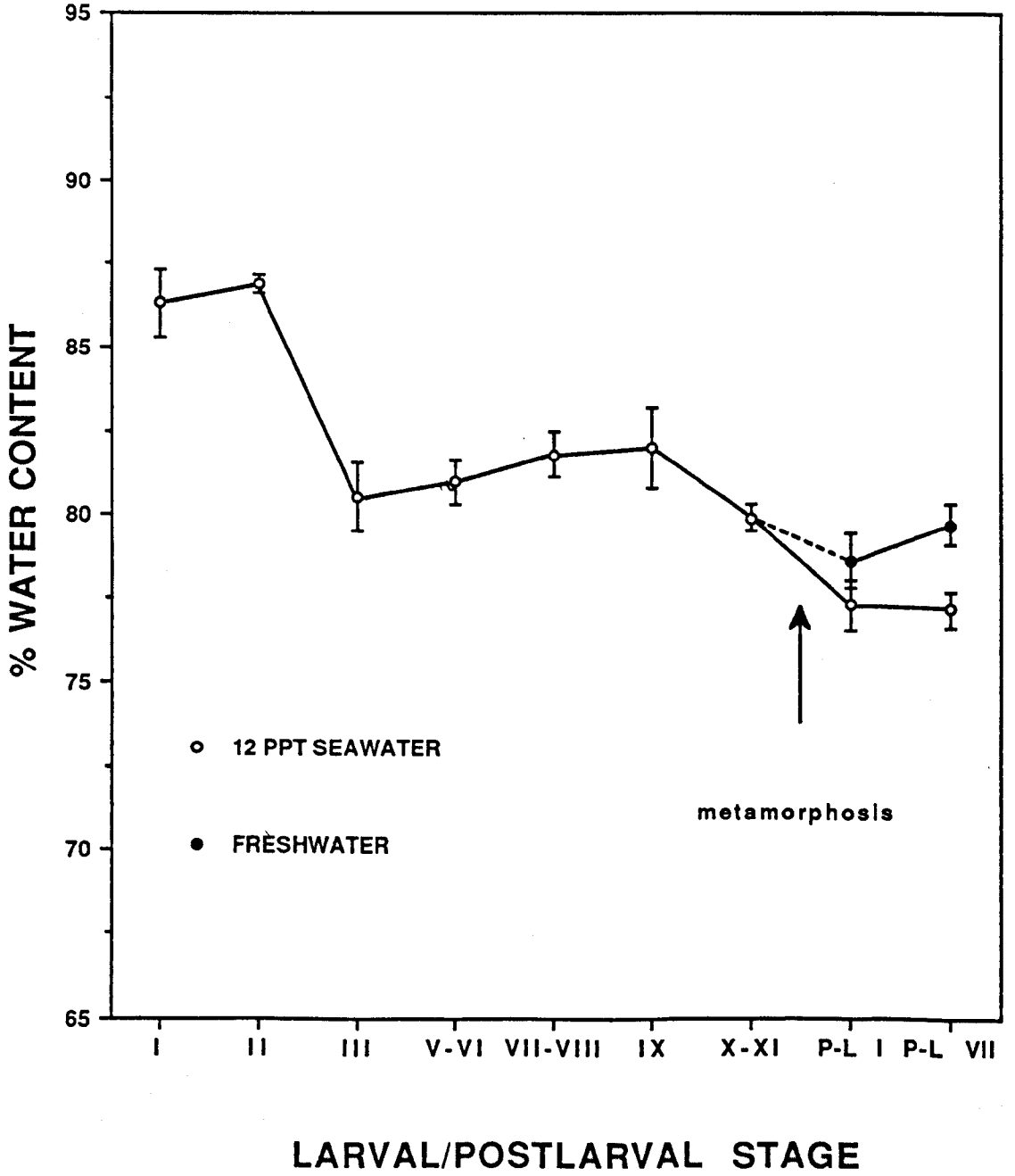
The first two larval stages had much higher water contents than the following larval stages. The percentage water content of the larvae remained approximately the same between stages III and IX. At stages X-XI there was a decrease in the water content towards the postlarval value. The postlarvae had different water contents depending upon whether they were held in fresh or brackish water. (Table XXX). The postlarvae maintained in freshwater had a higher water content than those in 12°/ooS seawater. This was to be expected, since the osmotic gradient between the animal and the medium was so much greater then that found in brackishwater. This different water content is interesting for it suggests that postlarvae maintained in freshwater would be heavier than their siblings in brackishwater. This was found to be the case when samples of the postlarvae were weighed.

Table (XXX) Wet weights of *Macrobrachium rosenbergii* postlarvae held in fresh and brackishwater. (Values expressed as mean ± s.d.).

	WET POSTLARVAL WEIGHT (mg)	
	12°/ooS seawater	freshwater
Postlarvae day 1	5.167 ± 0.144	6.218 ± 0.310
Postlarvae day 6	7.647 ± 0.567	9.585 ± 0.409

The wet weight determinations of postlarvae in fresh and brackishwater (Table XXX) showed that there was an immediate weight increase after 24 hours in

(Fig.40) The % water content of prawn larvae and postlarvae, reared in brackish and freshwater at 28 °C.



postlarvae held in freshwater over their siblings in brackishwater. This difference increased with time, the older postlarve having a greater weight difference. If the difference due to water content is removed, by studying the dry weights (Table XXXI), the differences between the larvae are not so great. However, they do show that the postlarvae maintained in freshwater were slightly heavier, and therefore probably larger.

Table (XXXI) Dry weights of *Macrobrachium rosenbergii* postlarvae held in fresh and brackishwater. (Values expressed as mean ± s.d.).

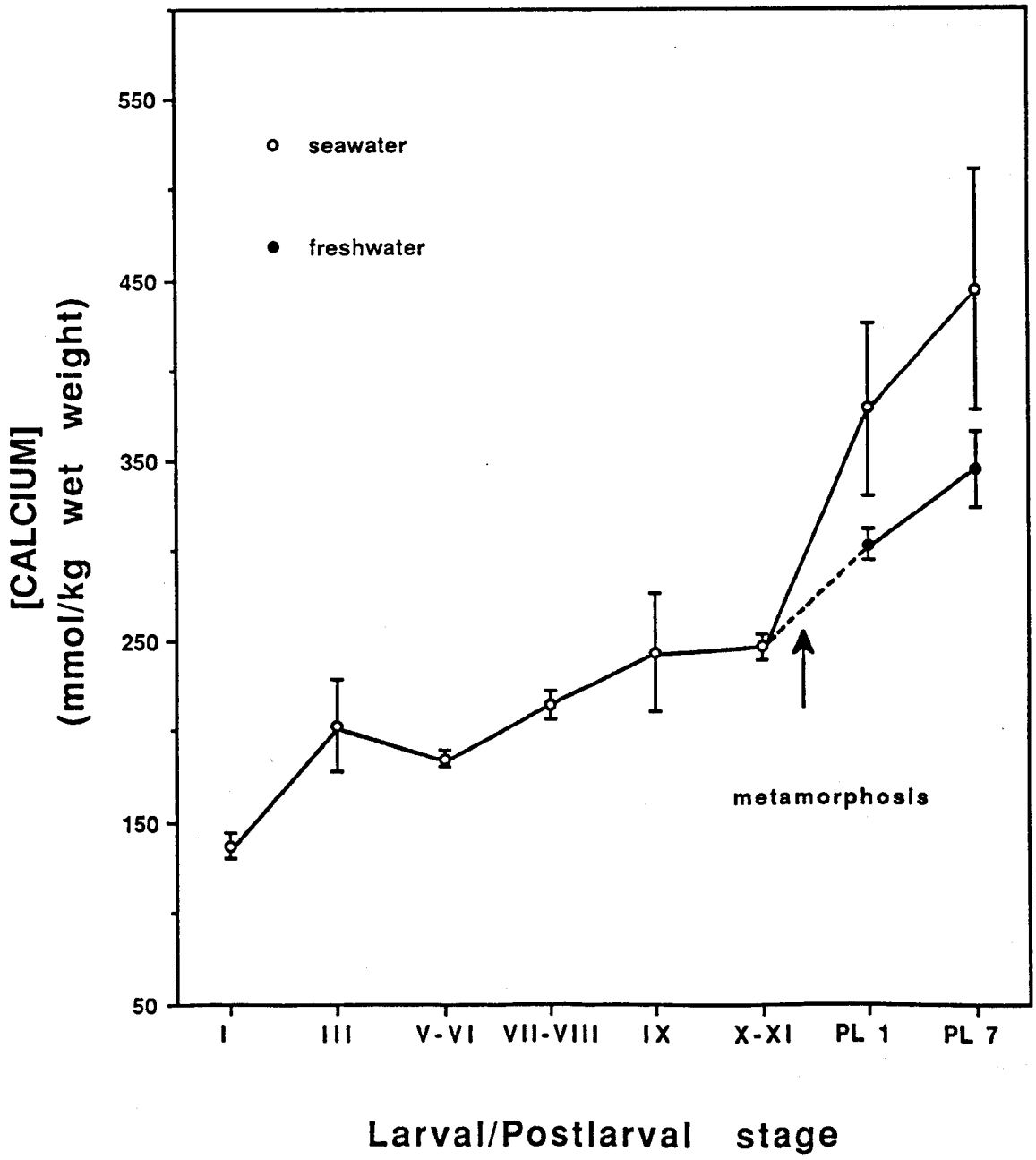
	DRY POSTLARVAL WEIGHT (mg)	
	12°/ooS seawater	freshwater
Postlarvae day 1	1.211 ± 0.021	1.364 ± 0.064
Postlarvae day 6	1.792 ± 0.058	1.872 ± 0.075

7.4.2.2 Calcium (Figs. 41,42).

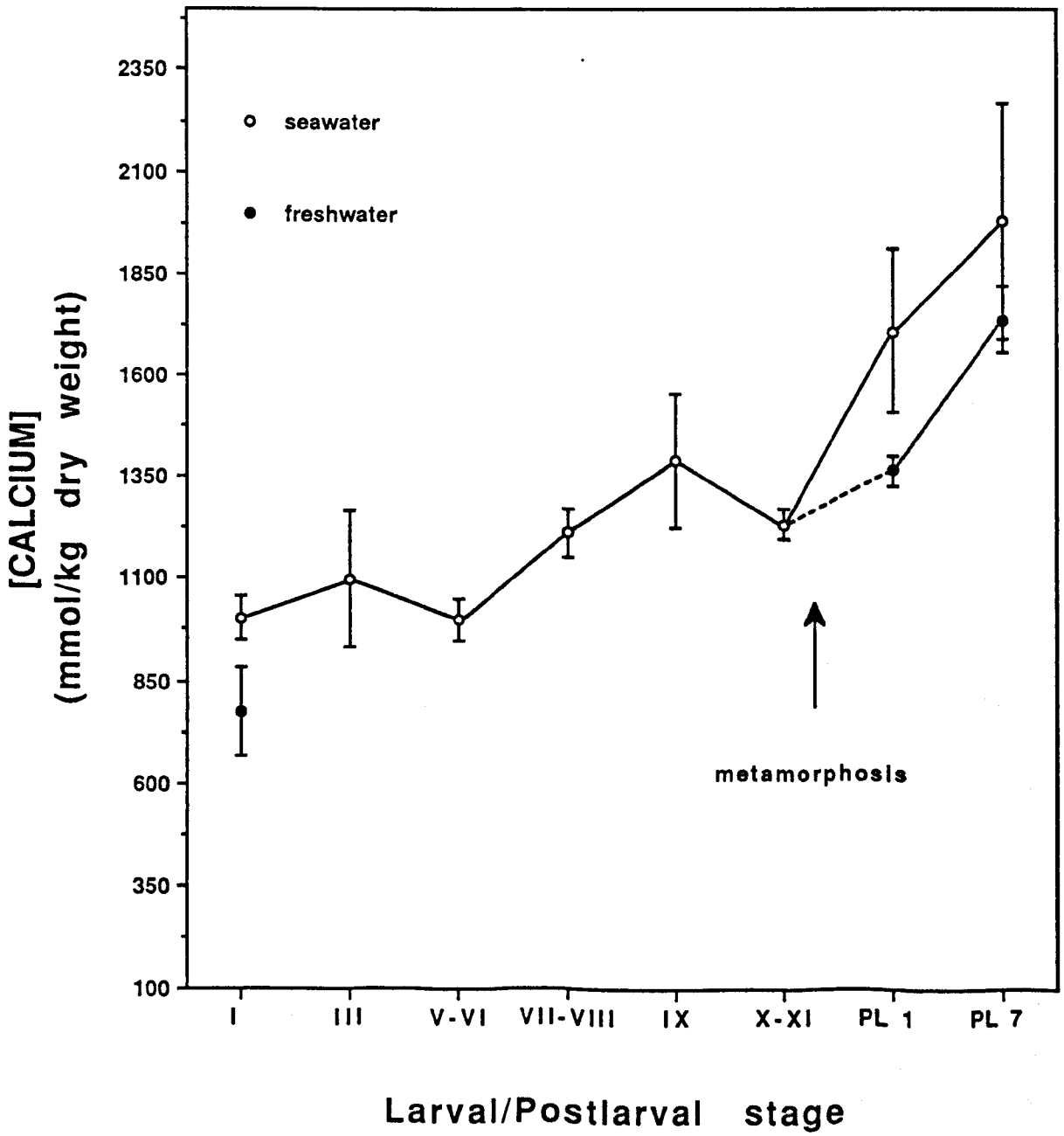
The wet weight calcium concentration of the larvae increased gradually throughout their development (Fig.41). The calcium concentrations ranged between 137 $mmol.kg^{-1}$ at stage I to 247 $mmol.kg^{-1}$ at stages X-XI. These calcium concentrations were 38 to 68 times higher than the environmental concentration at this salinity. The implication of this is that the calcium must be being concentrated in the larvae. This suggests that the larval cuticle is calcified because the cuticle is the principal site of calcium deposition in the adult. The gradual increase in the calcium content of the larvae on a dry weight basis (Fig.42) implies that either the cuticle becomes increasingly calcified as the larvae develop, or that the proportion of the larvae represented by the cuticle increases as the larvae develop. The latter hypothesis seems unlikely as the surface to volume ratio of the larvae would be expected to decrease as the larvae increase in size. This would have the effect of reducing the overall percentage of the total body weight represented by the carapace. However, the carapace might increase in thickness as the larvae develop. Thus the degree of calcification of the larvae might not change, but the total proportion of calcium would increase.

The calcium concentration continued to increase after metamorphosis. This

(Fig.41) The whole body [Ca] of prawn larvae and postlarvae, reared in brackish and freshwater at 28 °C. (Values expressed as mmol/kg wet weight).



(Fig.42) The whole body [Ca] of prawn larvae and postlarvae, reared in brackish and freshwater at 28 °C. (Values expressed as mmol/kg dry weight).



occured irrespective of whether the larvae were held in fresh or brackish water. The calcium content of the postlarvae maintained in freshwater was lower than that of their siblings maintained in seawater. This is interesting in that the dry weight of postlarvae maintained in freshwater is greater than that of postlarvae held in seawater (Table XXXI). Thus, the weight increase of the freshwater acclimated postlarvae does not seem to be due to an increase in the degree of calcification of the cuticle.

The difference between the calcium concentrations in stage I larvae held in fresh and brackishwater might be explained by the fact that the larvae were from different batches. Another explanation is that these larvae had not undergone a moult before being sampled and, as a result, their calcium concentration might be expected to represent that invested in the embryonic cuticle. If this is the case, then it shows that there is a difference in the degree of calcification of the larvae depending upon whether the eggs are incubated in fresh or brackishwater.

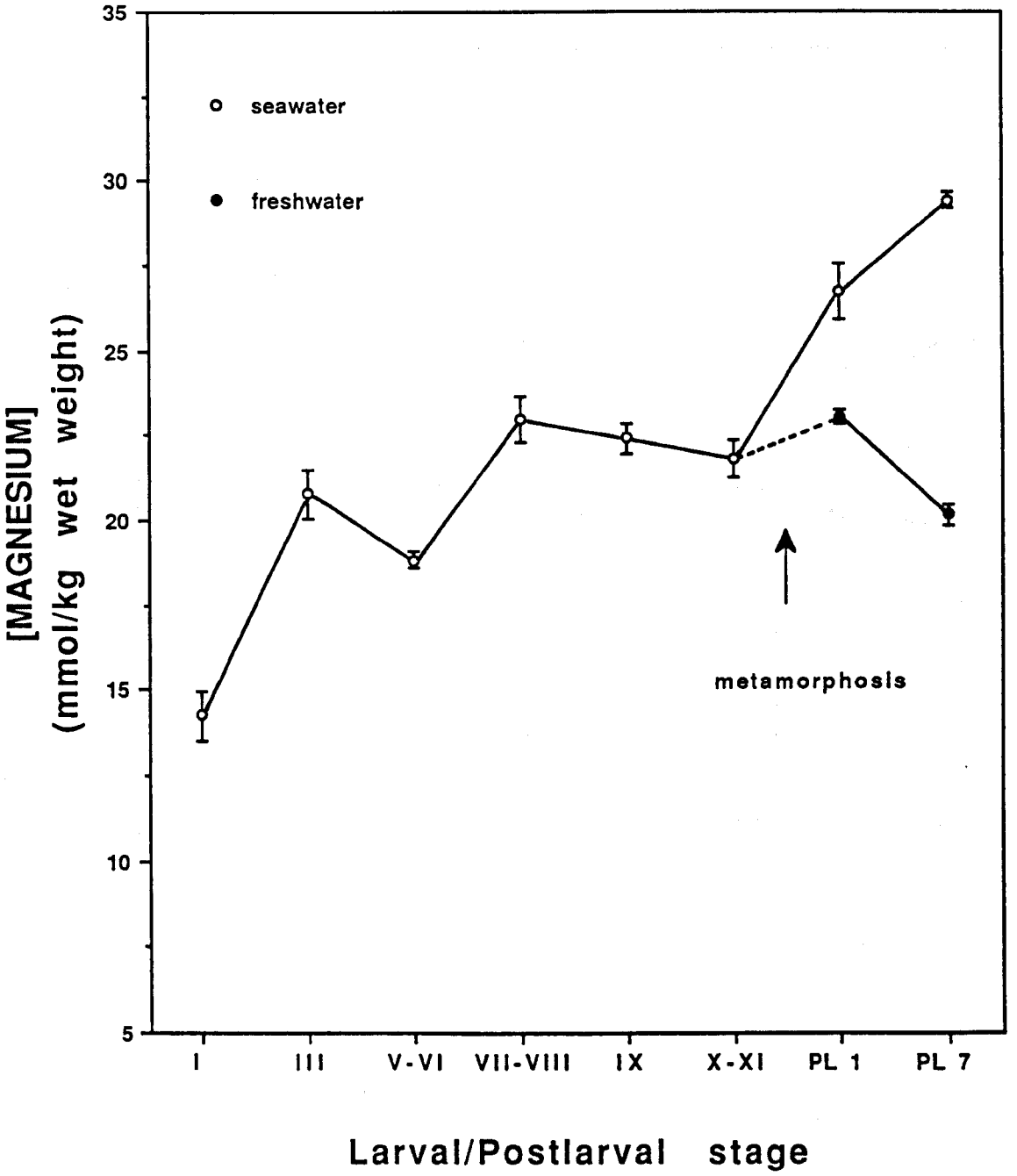
7.4.2.3 Magnesium (Figs.43,44).

The magnesium concentrations of the larvae did not provide a clear relationship between concentration and larval stage. The wet weight magnesium concentrations of the larvae showed a range of values between 18.9 - 23.0 mmol.kg^{-1} for the stage III to stage X-XI larvae (Fig.43). The stage I larva had a lower value at 14.3 mmol.kg^{-1}. The wet weight magnesium concentration in the larvae was similar to that found in the medium (12^{o}/oo = 18.3 mmol.kg^{-1}).

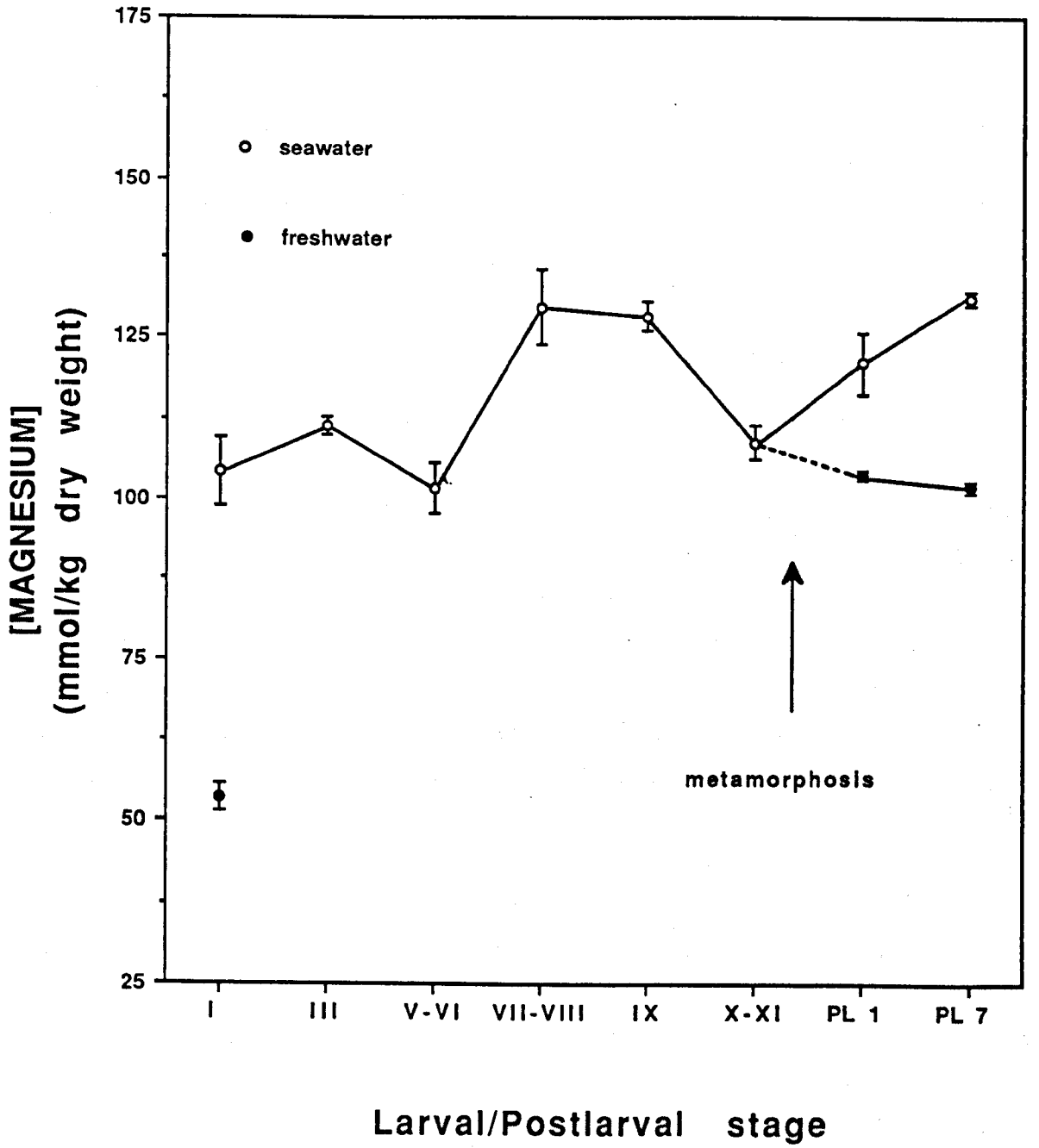
At metamorphosis there was an increase in both the wet and dry weight magnesium concentrations of the postlarvae held in brackishwater. There was a decrease in the magnesium concentration in the postlarvae held in freshwater after seven days.

The dry weight magnesium concentrations showed an interesting difference between the stage I larvae held in fresh and brackish water (Fig.44). The larvae that were hatched from eggs held in brackishwater had a magnesium concentration approximately double that (104.2 mmol.kg^{-1}) of those larvae hatched from eggs incubated in freshwater (53.5 mmol.kg^{-1}). The dry weight values do show that there was little difference between the stage I larvae in brackishwater and the stage III larva. Thus, the difference in their

(Fig.43) The whole body [Mg] of prawn larvae and postlarvae, reared in brackish and freshwater at 28 °C. (Values expressed as mmol/kg wet weight).



(Fig.44) The whole body [Mg] of prawn larvae and postlarvae, reared in brackish and freshwater at 28 °C. (Values expressed as mmol/kg dry weight).



wet weights is attributable to the different water contents of the larvae. The dry weight concentrations also show that the postlarvae in brackishwater do not increase their magnesium appreciably over that of the larvae. However, there still remains a difference between those postlarvae in fresh and those in brackishwater.

The range of dry weight magnesium concentrations for the larvae and postlarvae held in brackishwater was 101.7-131.2 $mmol.kg^{-1}$. These variations might be due to slight differences in the medium concentration of magnesium between the treatments. This is unlikely though, since the seawater used was an artificial formula. If the salinity was different due to inaccuracy on the part of the refractometer (or user), this would yield a medium magnesium concentration between 16.7 and 19.7 $mmol.kg^{-1}$(11-13°/ooS). This represents a difference of 17% in the magnesium concentration but the difference in the dry weight magnesium values for the larvae and postlarvae was 31%.

The wet weight magnesium concentrations for the stage III-XI larvae lay within the range 17.3-22.5 $mmol.kg^{-1}$ and this corresponded quite closely to the medium concentration of 18 $mmol.kg^{-1}$. This suggests two possibilities. Firstly, the larvae could have been conforming to the medium magnesium concentration, and had a negligible magnesium concentration in the carapace. Alternatively, the larvae could have been regulating the magnesium concentration at the same low level as the adults, and the rest of the magnesium was present in the carapace. The second hypothesis seems more likely since there was not an appreciable difference in dry weight magnesium concentration between the larvae and postlarvae. This suggests that the magnesium composition of postlarvae was similar to that of the larvae. The postlarvae, especially those acclimated to freshwater, were likely to have a magnesium composition corresponding to the adult state and thus by inference, so were the larvae. Another reason supporting the second hypothesis is that, if the larvae were conforming to the environmental concentration of magnesium, their body fluids would reflect the medium concentration, and the calcified cuticle would contain a high proportion of magnesium. Thus the total wet weight magnesium concentration of the larvae would be greater than the magnesium concentration of the medium. This high magnesium concentration would be lost upon metamorphosis, or when postlarvae were transferred to freshwater. Since this did not occur then it can be concluded that the magnesium concentration in the larvae was primarily due to the cuticle magnesium content, and the tissue concentration was probably

quite low.

7.4.2.4 Strontium (Figs.45,46)

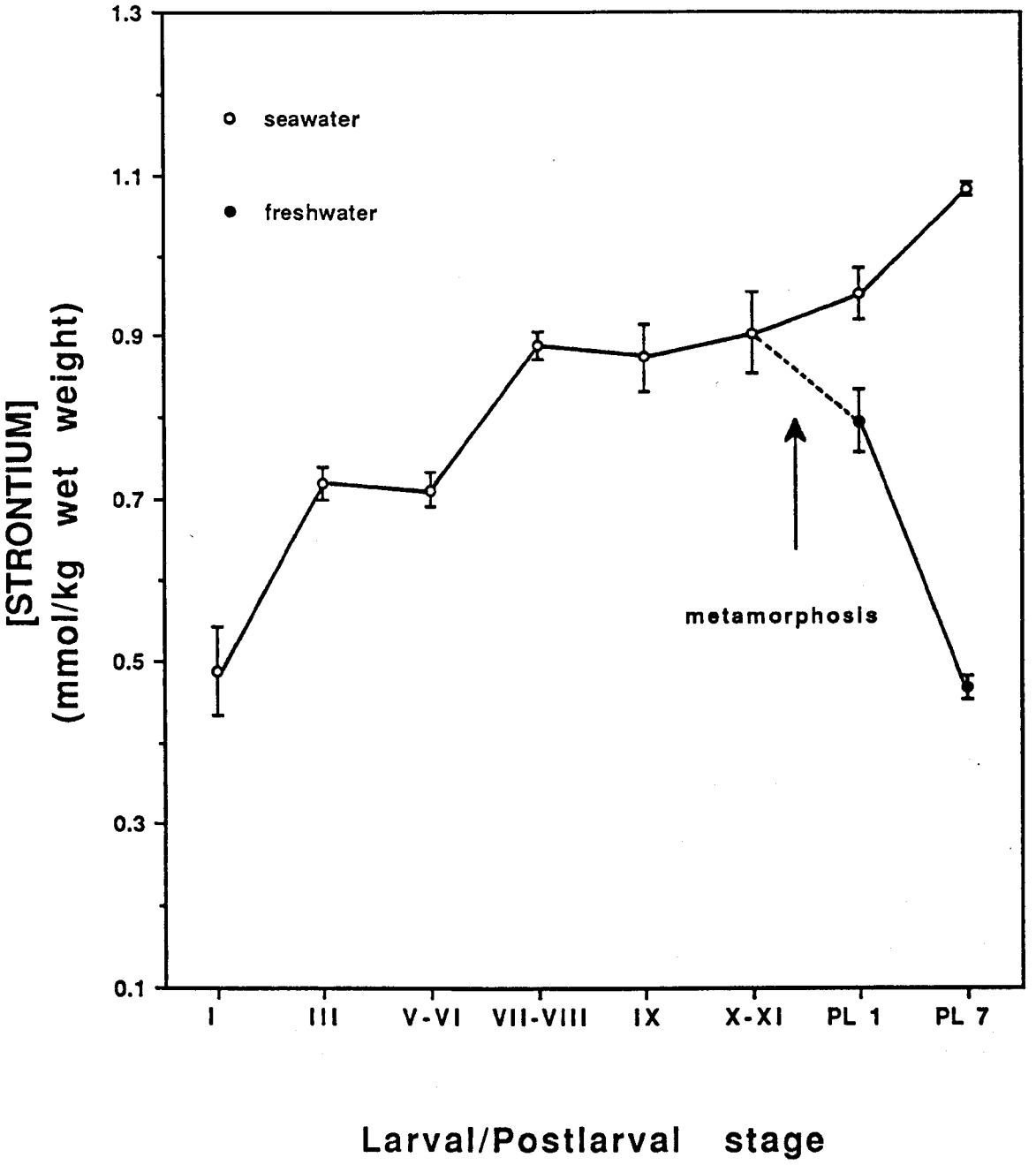
The wet weight strontium concentrations increased gradually during the course of larval development (Fig.45). At metamorphosis, there was a large divergence between those postlarvae maintained in freshwater and those held in brackishwater. The postlarvae held in freshwater lost a large proportion of their strontium after seven days, whilst the postlarvae held in brackishwater continued to increase their strontium concentration.

The wet weight concentration of strontium in the larvae ranged between 0.49-0.90 $mmol.kg^{-1}$. This was far greater than the environmental concentration of strontium which was approximately 0.029 $mmol.kg^{-1}$, and thus represents a concentration factor range of between 16.7-30.7.

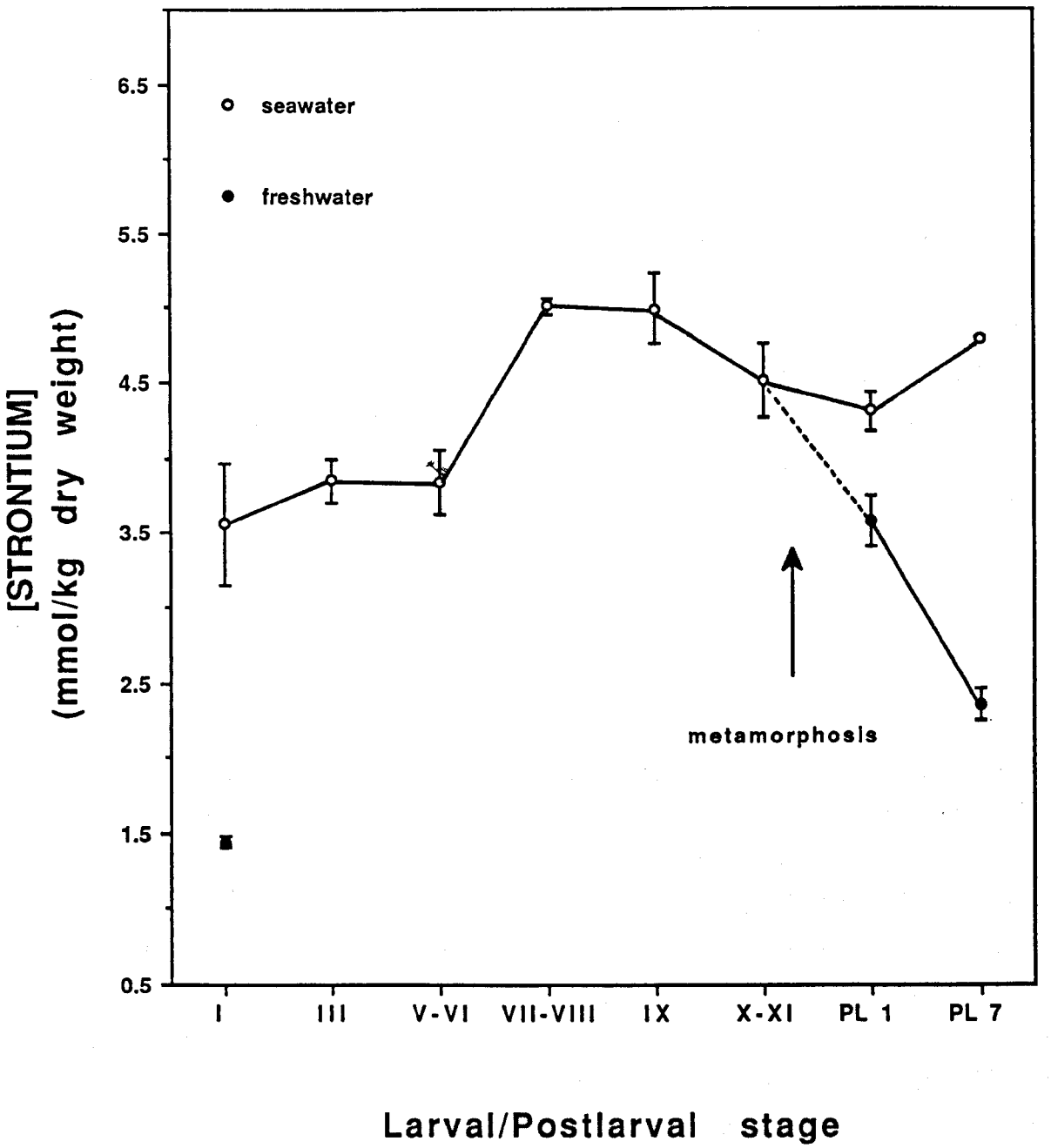
The dry weight strontium concentrations showed similar trends, except that the postlarvae in brackishwater had the same strontium concentration as the larvae (Fig.46). This is not surprising, and implies that the larvae and postlarvae regulate their strontium in a similar manner, the differences in wet weight concentration being attributable to water content differences. There was a large increase in strontium concentration between stages V-VI and stages VII-VIII. This might have been due to different strontium concentrations in the rearing water, but the size of the difference makes this unlikely.

The dry weight strontium concentration of the stage I larvae held in freshwater was very low, being under half (1.44 $mmol.kg^{-1}$) the concentration of those stage I larvae hatched in brackishwater (3.56 $mmol.kg^{-1}$). The elevated strontium concentrations in the larvae, above that of the medium, were presumably due to its incorporation in the carapace. The relatively stable concentration found in the larval and postlarval stages reared in seawater suggests a constant level of incorporation. However, the calcium concentration of these stages also increased, and thus strontium was forming a smaller percentage of the carapace composition as the larvae developed.

(Fig.45) The whole body [Sr] of prawn larvae and postlarvae, reared in brackish and freshwater at 28 °C. (Values expressed as mmol/kg wet weight).



(Fig.46) The whole body [Sr] of prawn larvae and postlarvae, reared in brackish and freshwater at 28 °C. (Values expressed as mmol/kg dry weight).

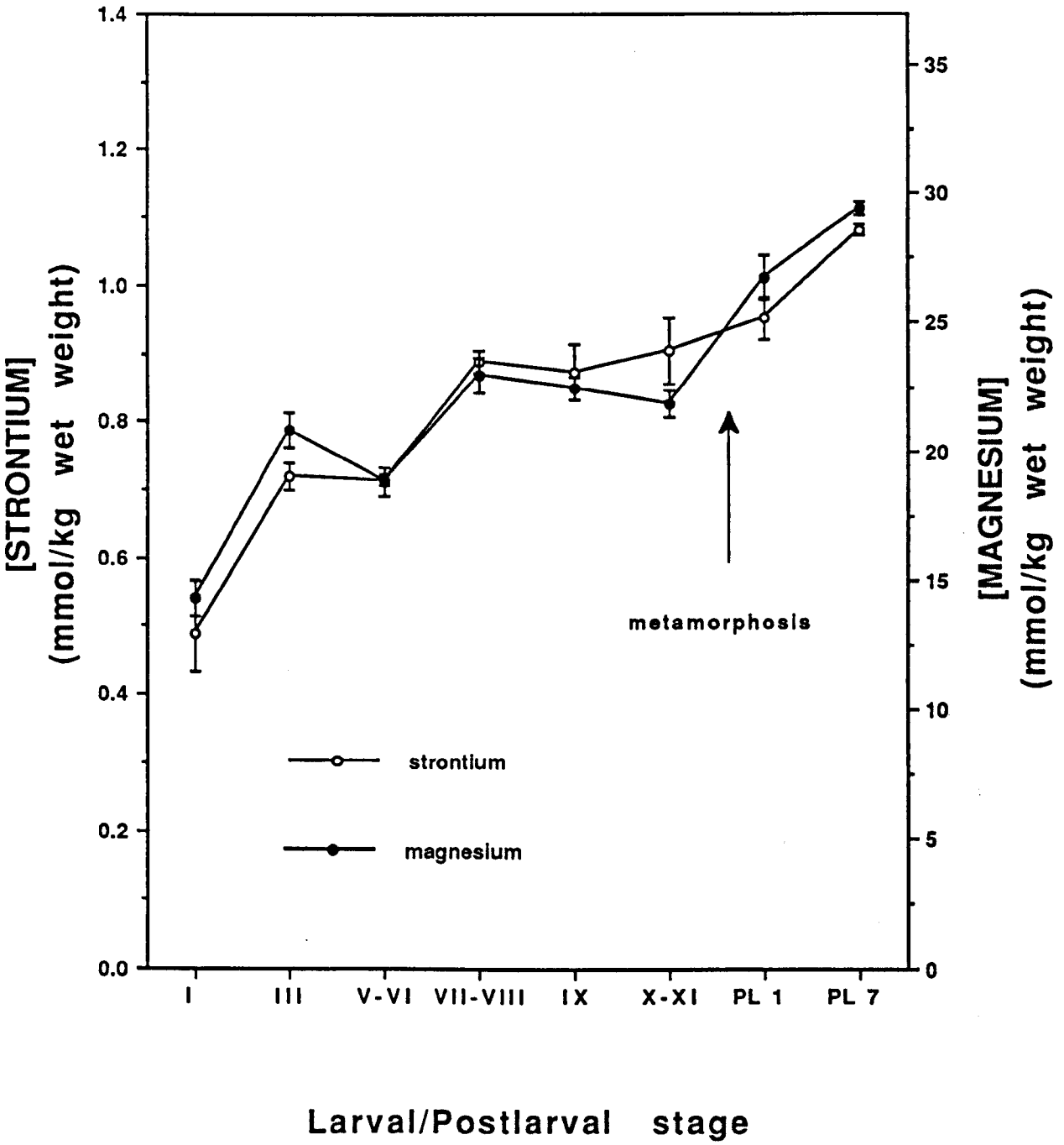


The environmental concentration seems to play a role in determining the strontium concentration of the larvae. This is evident from the stage I larvae and postlarvae held in freshwater. These stages had very reduced strontium concentrations, but in the adult haemolymph, strontium is well regulated over the range of salinities between fresh and 12 °/oo seawater. The reduction in the strontium concentration in the postlarvae cannot be attributed to a reduction in the haemolymph concentration. It is possible that the incorporation of strontium into the carapace is one method by which strontium excretion is regulated. This was suggested by Wickins (1984) for magnesium regulation in *Penaeus monodon.* If this were the case, then transfer to freshwater would reduce the available strontium and its removal to the carapace would also be reduced. The implication of this is that the uptake mechanism for strontium is not controlled, it merely imports all available strontium to the animal. Once the strontium is within the animal, homeostatic mechanisms maintain the desired concentration by excretion or removal to the carapace. This might also allow the remobilisation of the stored strontium should insufficient be available from the environment. This use of the carapace as a storage mechanism would be desirable if the prawn has a requirement for strontium. Since strontium is strongly regulated (section 6.2.2.9) it seems likely that the prawns do have a requirement for strontium at a concentration equivalent to that found in 12 °/oo seawater and maintenance of this haemolymph concentration in freshwater might require a storage mechanism.

7.4.2.5 Calcium, strontium and magnesium (Figs.47,48,49).

Superimposing the strontium and magnesium graph for larvae and post larvae maintained in brackishwater (12°/oo), in a similar manner to that shown in section 6.2.2.10, reveals a direct relationship between the concentrations of the two ions (Figs.47,48). There appears to be a mean Sr:Mg ratio of 1:26.8 . This ratio is lower than that found between strontium and magnesium in the adult haemolymph (1: 53, section 6.2.2.10). This direct relationship is curious as the two ions are less related to each other than they both are to calcium. However, if the larval, and brackishwater postlarval concentrations of either magnesium or strontium are plotted against calcium, a different relationship is observed (Fig.49). There is an exponential increase in the calcium concentration relative to magnesium and strontium. Thus it would seem that calcium is playing an increasingly major role in the calcification of the carapace relative to both

(Fig.47) The relationship between [Sr] and [Mg] in larval and postlarval prawns, reared in brackishwater at 28 °C. (Values expressed as mmol/kg wet weight).



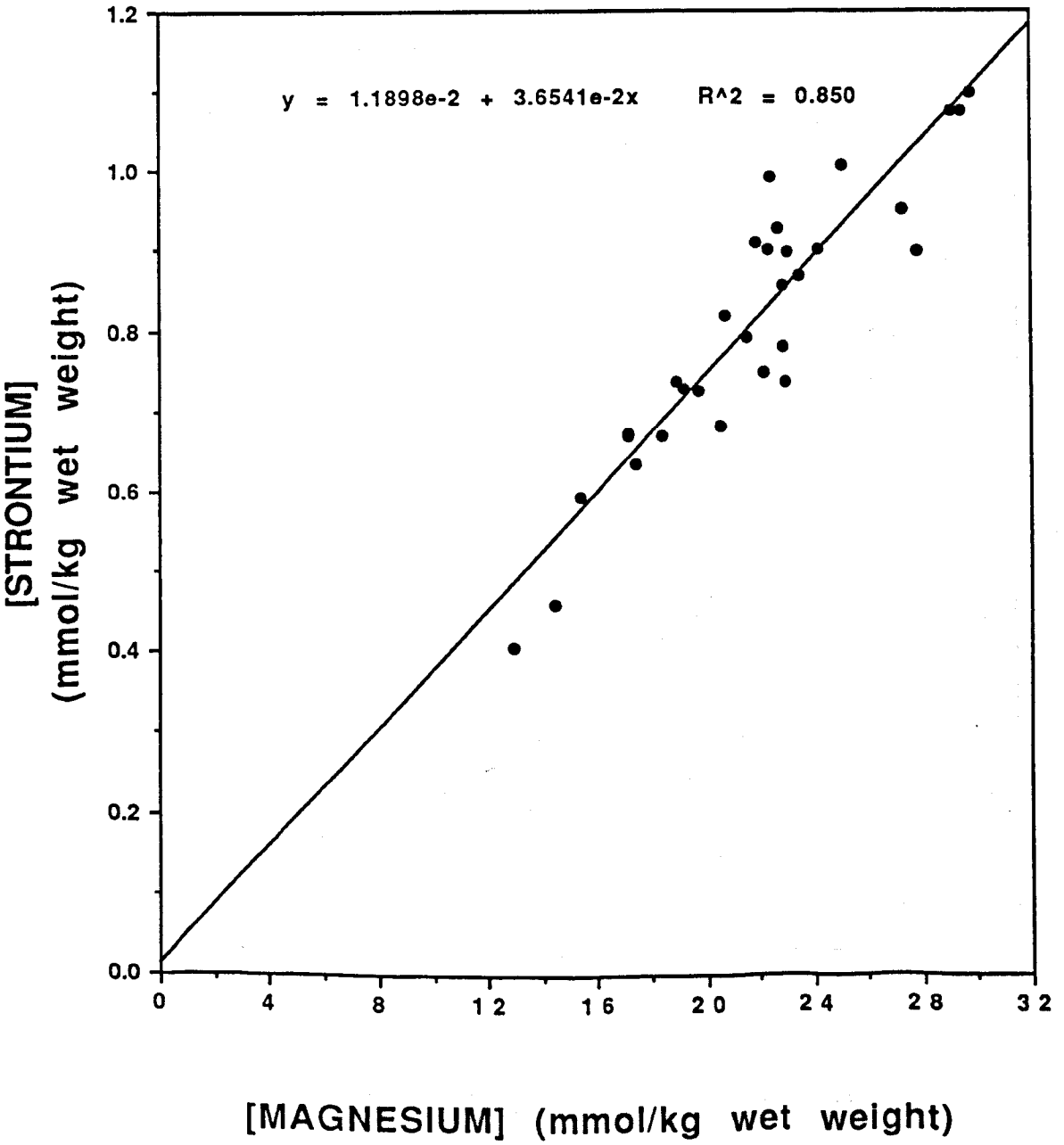
strontium and magnesium, and that these two elements remain in fixed ratio with each other in both larvae and postlarvae maintained in brackishwater.

The relationship between strontium and magnesium is retained in the postlarvae exposed to freshwater for 24 hours, and this is to be expected. The reason for this is that within 24 hours the majority of the postlarvae would not have moulted, and therefore their carapace composition would not be dissimilar from that of the previous stages exposed to seawater. However, after seven days in freshwater all of the postlarvae would have moulted, and thus the decrease in strontium concentration becomes evident. The ratio between strontium and magnesium in postlarvae maintained in freshwater for seven days is different to that found in brackishwater maintained postlarvae. This is principally due to the large decrease in the strontium concentration of the postlarvae.

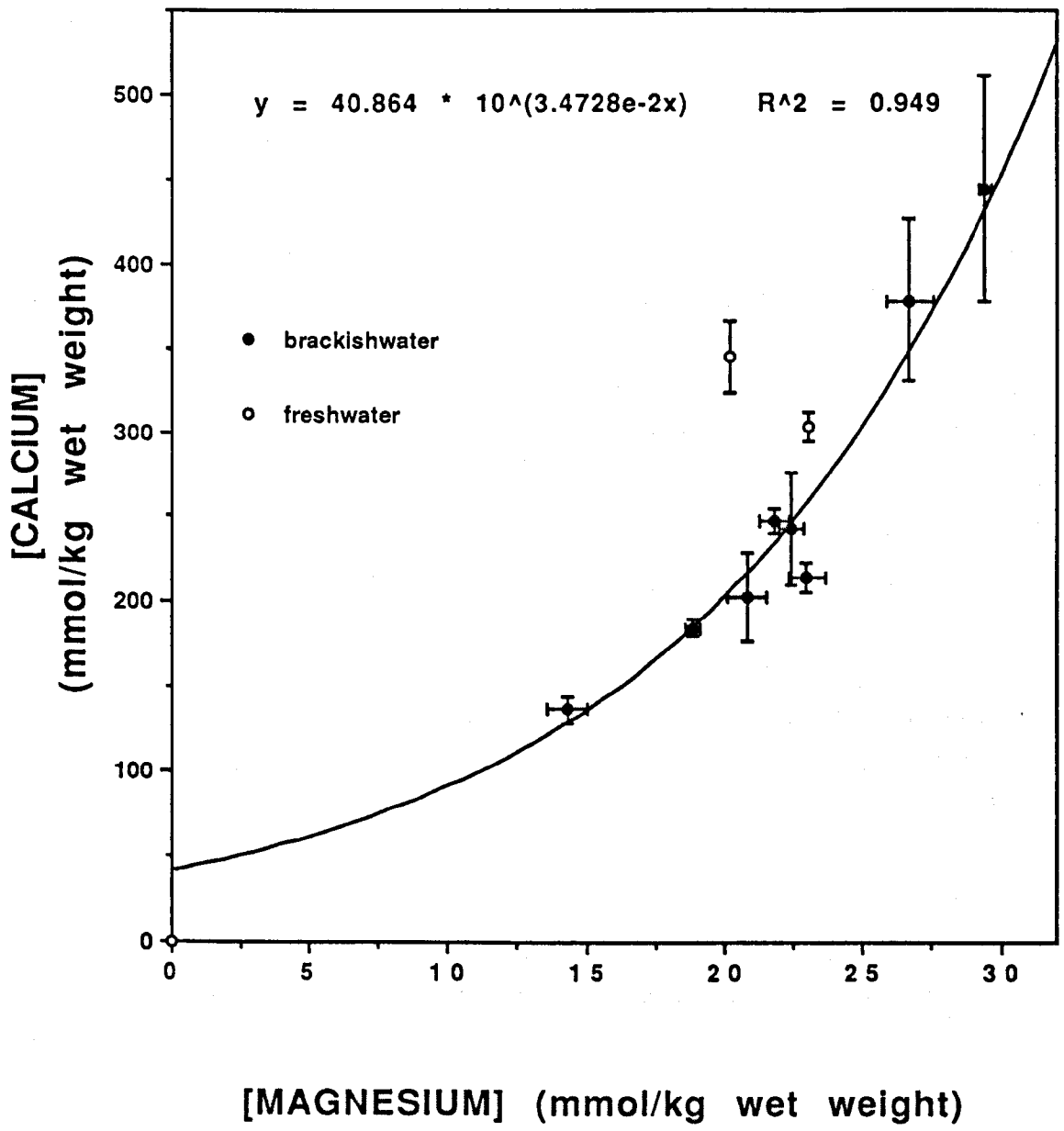
The possible reason for this decrease in strontium concentration in postlarvae held in freshwater is difficult to explain. From adult data it is known that the haemolymph strontium concentration is strongly regulated in freshwater at a concentration corresponding to that found in 12^{o}/oo seawater. Thus the decrease in strontium concentration of the carapace would be unlikely to be due to a decrease in haemolymph concentration of the ion. It is known that the environmental concentrations of strontium, magnesium and calcium affects their degree of incorporation into calcified carapaces (section 7.2.2), and that substitution of calcium by magnesium and strontium occurs in aragonite and calcite. Thus the decrease in the strontium concentration of the carapace might be due to decreased substitution into the crystal structure from the external medium.

In freshwater, the environment cannot play a significant role in contributing elements to the carapace by means of substitution into the crystal lattice. The result of this is that cuticle calcification must be entirely derived from internal sources of calcium, magnesium and strontium. The reduction in the strontium concentration of the carapace of postlarvae in freshwater might be due to a closer cycling of the element within the tissues of the prawn. This would reduce the need to deposit strontium in the carapace as a means of excretion. The reason why total strontium concentration is reduced in postlarvae transferred to freshwater is probably a function of both effects. Although the strontium concentration of the larvae is decreased by 50% when exposed to freshwater for seven days, the total concentration is still far higher than the environmental

(Fig.48) The relationship between [Sr] and [Mg] in larval and postlarval prawns reared in brackishwater at 28°C. (Values expressed as mmol/kg wet weight)



(Fig.49) The relationship between [Ca] and [Mg] in larvae and postlarvae, reared in brackish and freshwater at 28 °C. (Values expressed as mmol/kg wet weight)



concentration, and even the concentration found in 12°/oo seawater. Can this decrease be attributed to a reduction in the substitution of strontium into the crystal lattice?

It is possible that the effect of the freshwater habitat changes the manner in which calcification occurs, and that the new concentration of strontium reflects an altered solubility for the ion. This would be surprising, as in freshwater the calcification is occurring from internally derived products, and the adult prawns' homeostatic mechanisms appear to be capable of maintaining the same internal ionic environment when exposed to salinities between 0-12°/oo.

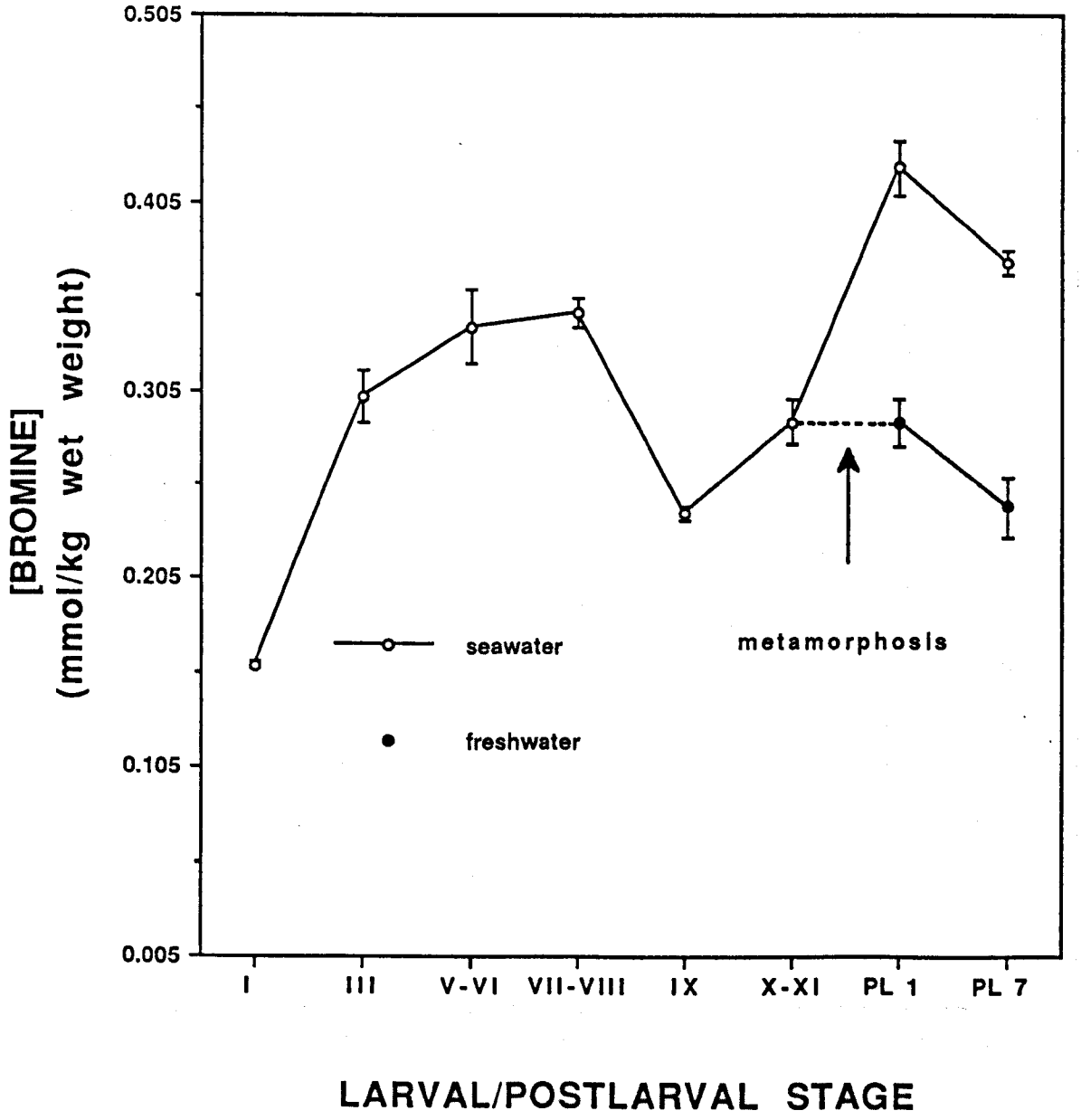
7.4.2.6 Bromine (Figs.50,51).

The graphs of the whole body bromine concentrations of the larvae do not present the clear relationships observed for strontium, magnesium and calcium. There appears to be a gradual increase in the larval bromine concentration from stage I to stages VII-VIII. The bromine concentration of the stage I larvae in freshwater is lower than that found in the stage I larvae hatched in freshwater (difference = 24%) (Fig.51). There is a large decrease in the bromine concentration between stages VII-VIII and stage IX. The bromine concentrations of the larvae when expressed as wet weight values are in the range of the bromide concentration of the seawater in which the larvae were being reared.

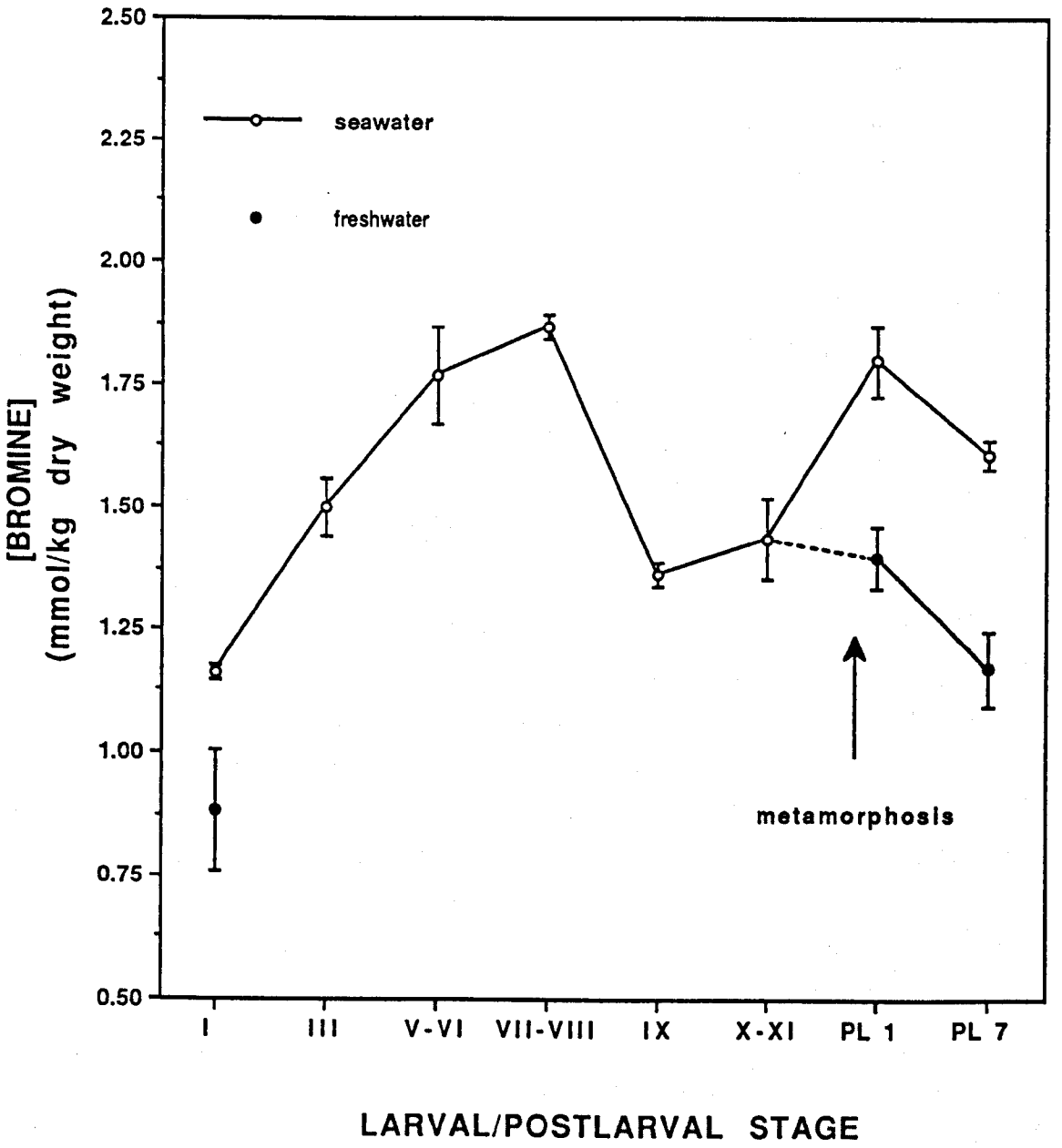
Since these larval bromide concentrations are being expressed as whole body concentrations no account is being taken for possible partitioning of the bromine within the larvae. Analysis of the exuvia of stage IX larvae, collected from the surface of the rearing tank revealed a bromine concentration of between 13.9-14.7 $mmol.kg^{-1}$ of dry material (1107-1171 ppm). Thus it is evident that a considerable amount of bromine is present in the exoskeleton. This is in agreement with the conclusions of other workers who maintain that tissue concentrations of bromide are approximately equal to the medium concentration, but that the sclerotised cuticle contains high concentration of organically bound bromine (section 5.2).

Larval stages IX, X-XI, and the seawater acclimated postlarvae were all maintained in the same seawater, and thus the concentration of bromide in the seawater cannot be cited as a cause for the difference in concentrations observed between these

(Fig.50) The whole body [Br] of prawn larvae and postlarvae, reared in brackish and freshwater at 28 °C.(Values expressed as mmol/kg wet weight).



(Fig.51) The whole body [Br] of prawn larvae and postlarvae reared in brackish and freshwater at 28 °C.(Values expressed as mmol/kg dry weight).



stages. However, stages I, III, V-VI and VII-VIII were all reared independently in fresh batches of artificial seawater. The object of using artificial seawater for these rearing trials was that it enabled a reproducible medium to be used to try and avoid variations in trace element concentrations, especially bromine and strontium. Providing the salinity was the same in all the treatments the concentrations of all the ions should have been the same and irrespective of the salinity, the ratios between the ions would be constant.

It is possible that the bromide concentration of the seawater in the late stage larval rearing could have been depleted due to uptake by the larvae. However, it seems unlikely that such a large reduction in the medium concentration could have taken place between stages VII-VIII and stage IX. What is more probable is that the initial concentration of bromide in the medium was lower in the stage IX and postlarval treatment. This explanation assumes that the incorporation of bromine/bromide into the larvae is occurring as a function of the environmental concentration. Thus a reduced environmental concentration will be reflected in a reduced larval concentration. This follows the hypothesis of Hunt (1984). There is some evidence for this in the postlarvae acclimated to freshwater, whose bromine concentration decreases with time. However, the freshwater acclimated postlarvae still contain appreciably high concentrations of bromine. This suggests that the cuticle still contains some brominated scleroproteins. This contradicts Hunt's (1984) hypothesis that brominated scleroproteins are replaced by chlorinated ones in freshwater species. The high haemolymph bromide/bromine concentrations maintained by adult *Macrobrachium rosenbergii* maintained in freshwater could provide the bromine required for incorporation into the cuticle. If this is the case then the implication is that bromine/bromide has some function in the correct formation of the carapace. The source of the bromide that is incorporated into the carapace is the haemolymph, this acts as a transporting medium for the constituents that form the carapace (section 7.2.2). It is possible that, if the haemolymph perfuses the cuticle to deliver the organic and inorganic components of the cuticle, bromide is delivered in a similar manner. Thus, when sclerotisation occurs the bromide is present in the carapace and could be oxidised and incorporated into the scleroproteins. This is in agreement with Hunt's (1984) hypothesis that bromine incorporation is a secondary effect of sclerotisation. The gradual increase in the haemolymph bromide concentration of adult prawns acclimated to increasing salinities indicates a possible method by which the environmental bromide concentration might be reflected in the cuticle concentration. The incorporation of bromine into the cuticle as it is developed and sclerotised may partially

explain the reduction in haemolymph bromide concentration during premoult and postmoult (section 6.3.2.6, Fig.37). If this is occurring a gradual reduction in the bromine concentration might be expected during the premoult and postmoult period, rather than the relatively constant low concentration observed.

The graphs of larval strontium and magnesium concentrations do show a slight reduction between stages VII-VIII and stage IX. Calcium was not reduced between these stages but was actually increased. This suggests that the reduction in bromine concentration might have been due to a shift in the emphasis on cuticle hardening away from sclerotisation towards calcification. This explanation does not account for the increase in bromine concentration of the larval and postlarval stages that followed.

The increase in bromine concentration that occurred between stage X-XI larvae and postlarvae maintained in seawater is in accordance with the increase in the calcium, magnesium and strontium concentrations. However, the calcium, magnesium and strontium concentrations continued to increase from PL I to PL VII, whereas the bromine concentration was reduced.

7.5 Conclusion

There does not appear to be a change in the mechanism of cuticle hardening between the larval and postlarval stages of *Macrobrachium rosenbergii*. This is apparent from the high concentrations of calcium, strontium, magnesium and bromine detected in the larvae. Although the larvae and postlarvae share the same calcification mechanism there are differences between the various stages. It seems that the postlarvae are slightly more calcified than the larval stages preceeding metamorphosis. It is unclear whether this increase in calcium concentration is sufficient to explain the change from a planktonic to benthic lifestyle that occurs between the larva and postlarvae.

There does not appear to be a difference between the compositions of stage I and stage II larvae. This suggests that if there is a permeability difference between the stages it is not connected to either calcification of sclerotisation.

The large increase in the bromine concentration of the larvae that occurs between the first five larval stages might explain the requirement for bromide found in sections 5.4.1 and 5.4.2. The high concentration of bromine in the postlarvae acclimated to freshwater for seven days suggests that its incorporation into the carapace occurs via the transport of haemolymph products to the cuticle. It is known that the tegumental glands contain many of the products associated with sclerotisation (7.1), but whether they contain ionic bromide derived from the haemolymph, or whether the bromine is already contained within brominated phenolic compounds must be the subject of further work.

If bromide is essential for the correct functioning of the sclerotisation process, then this is a considerable clue as to the possible role of bromide generally in physiology. The metabolic pathways that lead from tyrosine to scleroproteins or melanins via the catecholamine compounds are found throughout nature. The blackening of cut potatoes, other vegetables and fruit in air is caused by the action of phenol oxidase enzymes. This oxidation is partially prevented by the presence of vitamin C. It has been found that chloride and bromide can prevent this occurring as well. In many animals the catecholamines are neurohormones and the dysfunction of the processes that control these neurohormones is implicated in many nervous diseases. This might partially explain why massive doses of bromide has an effect on nervous activity in humans.

CHAPTER 8 - Salinity Effects on Respiration.

8.1 Introduction

Macrobrachium rosenbergii encounters variation in environmental salinity at several stages of its lifecycle. The duration of the larval part of the lifecycle is spent entirely in brackishwater, a biotope characterised by a fluctuating salinity regime. After metamorphosis the postlarvae of this species begin to enter freshwater, a transition that may last for several weeks. However, it is possible to transfer newly metamorphosed postlarvae directly from brackishwater (12°/ooS) into freshwater (pers. obs.). As adults, the spawning migration made by the female from freshwater towards brackishwater may expose her to a fluctuating salinity regime. Some populations of *Macrobrachium rosenbergii* are known to inhabit brackishwater, and this and other *Macrobrachium* species have been cultured in brackishwater (Johnson 1967; George 1969; Perdue & Nakamura 1976; Smith *et al.* 1982).

The ability of the adult *Macrobrachium rosenbergii* to regulate their internal osmotic and ionic environment has been considered in previous sections. More specifically the effect of salinity on their iono- and osmoregulatory ability has been studied (section 6.0). Variations in environmental salinity can affect the respiratory physiology of *Macrobrachium rosenbergii* in five ways: by affecting gas solubility in the water pumped over the gills; by affecting the solubility of gases in the plasma; by directly affecting the oxygen affinity of haemocyanin; by causing an altered pH in the haemolymph, which also affects the oxygen affinity of haemocyanin; and by causing a change in haemolymph haemocyanin concentration (Cameron & Mangum 1983).

The principal buffering substances in crustacean haemolymph are HCO_3^- and proteins (including haemocyanin) (Cameron & Mangum 1983). If the pH of the blood of aquatic animals decreases due to a respiratory acidosis there appears to be a compensatory mechanism which increases the concentration of HCO_3^- in the blood. This causes an increase in blood pH in an attempt to return it to the normal resting value (Henry *et al.* 1981). The method by which the HCO_3^- is controlled is thought to be by exchange with Cl^- across the gills (and/or Na^+ for H^+) (Henry *et al.* 1981). In the

terrestrial crab, *Gecarcinus lateralis*, the use of Cl^-/HCO_3^- exchange is not possible since it is not immersed in water, and thus it was suggested that these crabs utilise the dissolution of carapace $CaCO_3$ to provide the necessary HCO_3^- ions (Henry et al. 1981). There is a corresponding increase in haemolymph Ca^{2+} as a result of this dissolution. However, Cameron (1985) found that carapace dissolution provided only 7.5% of the carbonates/bicarbonates required for the compensation of haemolymph acidosis in *Callinectes sapidus*. His conclusion was that the medium provided the bulk of the bicarbonate required by this aquatic crab and that carapace dissolution was of secondary importance. An increase in the concentration of Ca^{2+} ions in the haemolymph was observed, but Cameron (1985) concluded that this was a temporary phenomenon caused by the acidification of the haemolymph, and restoration of the haemolymph pH resulted in the cessation of this effect.

In the shrimp *Crangon crangon* and the prawn *Palaemon adspersus* haemolymph chloride concentrations decreased under hypoxic stress (Hagerman & Uglow 1981,1982). It may be that this was a result of the increased inward transport of HCO_3^-. The haemolymph concentration of Ca^{2+} increased when *Crangon crangon* was exposed to dilute seawater ($10^o/ooS$), and might be due to carapace dissolution due to low environmental HCO_3^- concentration (Greenaway 1974; Hagerman & Uglow 1982). A reduction in environmental salinity will result in a reduced environmental HCO_3^- concentration, and this might explain the low tolerance of *Macrobrachium rosenbergii* to hypoxia when in freshwater. If the environmental concentration of HCO_3^- was low, the prawn might be forced to use carapace dissolution as a source of HCO_3^-. Alternatively there might be an unacceptable loss of Cl^- as the prawn attempted to extract HCO_3^- ions from the medium.

The pH of crustacean haemolymph affects the oxygen affinity of haemocyanin via the Bohr effect, an increase in the pH causing an increased oxygen affinity. The oxygen affinity of *Macrobrachium rosenbergii* haemocyanin was shown to be strongly pH dependent (Mauro & Malecha 1984b). These authors observed that *Macrobrachium rosenbergii* has a hyperventilatory response to environmental hypoxia. This has the effect of reducing the PCO_2 at the gills, thereby increasing the haemolymph

pH and allowing increased oxygen uptake by the haemocyanin (Mauro & Malecha 1984a). The increased ventilatory response is not maintained at very low oxygen tensions as the energy cost of ventilation becomes excessive.

The haemolymph pH of crustaceans in low salinity waters is considerably higher than that of crustaceans in higher salinity waters (Truchot 1973; Cameron 1978; Weiland & Mangum 1975; Mangum & Towle 1977; Morris *et al.* 1988). It has been suggested that this alkalosis is caused by an increase in the haemolymph concentration of NH_3, as a result of the deamination of proteins that occurs during low salinity acclimation. The NH_3 in the haemolymph removes protons due to the formation of NH_4^+ ions thereby increasing the pH (Gerard & Gilles 1972; Mangum *et al.* 1976). This high pH causes an increase in the oxygen affinity of haemocyanin and results in a relatively unchanged P_{50} over quite a wide range of salinities (Cameron & Mangum 1983). This process of maintaining a stable P_{50} was termed '*enantiostasis*' by Mangum & Towle (1977), and was considered to be of adaptive significance to a crustacean inhabiting an estuarine environment where hypoxia might be encountered in conjunction with salinity fluctuation. Wheatley & McMahon (1982b) studied the effect of hypersaline exposure on the euryhaline crayfish, *Pacifastacus leniusculus*. Under these conditions there is an increase in the ionic concentration of the haemolymph as well as an acidosis. However, they could not demonstrate a stable P_{50} over the salinity range 0-25.5^{o}/oo. These authors did find that the pre- and post-branchial oygen concentrations were approximately the same between 8.5-25.5^{o}/ooS. In freshwater, this species had substantially elevated pre- and post-branchial oxygen concentrations .

Macrobrachium rosenbergii is reported to have a high resting metabolic rate and a poor tolerance of acute hypoxia. This high metabolic rate is suggested to be a possible reason for the high growth rates observed in this species (Spotts 1983). Mauro & Malecha (1984) concluded that *Macrobrachium rosenbergii* is relatively tolerant of hypoxia thus contradicting the findings of Spotts (1983). The lowest dissolved oxygen concentration that can be tolerated by juvenile *Macrobrachium rosenbergii* for short periods is between 1-1.5 ppm Wulff (1982). A lack of tolerance of hypoxic conditions in ponds by this species has been reported and the prawns' response is usually migration to the shallower water at the edges of the ponds, and even emergence from the water

(emersion) (Willis & Berrigan 1976; T. Warren pers. comm. 1990). Partial emersion has also been observed in the intertidal prawn, *Palaemon elegans*, as a response to environmental hypoxia (Taylor & Spicer 1988). The advantage of this is that the higher partial pressure of oxygen (PO_2) in air, coupled with the solubility of CO_2 in seawater, may facilitate oxygen uptake and CO_2 excretion, thereby avoiding blood acid-base disturbance. By remaining in contact with the seawater these prawns are also able to withstand desiccation better than those prawns which are fully emersed.

Studies on the effect of salinity on the rate of oxygen consumption of postlarval, juvenile and adult *Macrobrachium rosenbergii* revealed that there was no significant difference in the rates between the salinities of 0-28°/oo (Nelson *et al.* 1976). In contrast an increase in temperature resulted in an increase in oxygen consumption (Nelson *et al.* 1976; Stephenson & Knight 1980). The energy cost of ionic regulation is suggested to be considerable (Taylor *et al.* 1977), and thus under simultaneous salinity and oxygen stress the ionic regulation of a crustacean may be affected.

The pH, P_{50}, n_{50}, Bohr effect and PO_2 of the pre- and post-branchial haemolymph have been studied in a number of crustaceans, and the data is summarised by McMahon & Wilkens (1983) and by Mangum (1983). It is difficult to generalise about the values obtained since differences exist due to the varying requirements of the individual species. Table XXXII presents haemolymph PO_2's together with *in vivo* pH and P_{50} data for some crustaceans whose habitats range between fresh and full strength seawater.

The values presented in Table XXXII show a wide variation in haemolymph PO_2, although it is interesting to note the low values recorded for the two freshwater species listed (*Orconectes rusticus, Astacus leptodactylus*), as well as the values for *Pacifastacus leniusculus*. The pH values range between 7.4-8.1 and the P_{50} values between 4-12 Torr. The post-branchial haemolymph always has a higher PO_2 than the pre-branchial haemolymph. The PO_2 values for post-branchial haemolymph are between 9 - 97 Torr, and the pre-branchial haemolymph values lie within a closer range of values

(3.3-18 Torr).

Table (XXXII) Oxygen transport characteristics, and *in vivo* pH values for the haemolymph of various decapod Crustacea.

Species	(P_aO_2) (Torr)	(P_vO_2) (Torr)	pH_a	P_{50} (Torr)	ref.
Homarus americanus	69	18	-	-	a
Homarus gammarus	49	-	7.80	6	b
Cancer magister	32	7	-	-	c
Cancer productus	51	13	7.84	-	d
Carcinus maenas	75	10	7.84	7	e
Carcinus maenas	97	18	-	-	f
Callinectes sapidus	35	14	7.59	12	g
Callinectes sapidus	64	12	7.67	-	h
Pacifastacus leniusculus	9	5	7.83	-	i
Astacus leptodactylus	11	7	7.44	6.2	j
Orconectes rusticus	19.7	3.3	7.78	7.8	k

a) McMahon & Wilkens (1975)
b) Butler *et al.* (1978)
c) Johansen *et al.* (1970)
d) deFur & McMahon (1978)
e) Taylor & Butler (1978)
f) Taylor (1976)
g) Mangum & Weiland (1975)
h) Mangum (1977)
i) Rutledge (1981)
j) Angersbach & Decker (1978)
k) Wilkens & McMahon (1982)

Haemocyanin increases the oxygen carrying capacity of the haemolymph, but also performs several other functions. These are, its buffering action in the haemolymph, its contribution to the haemolymph osmotic pressure (Redmond 1971; Cameron & Mangum 1983). Haemocyanin is also thought to act as an organic reserve that can be utilised during periods of starvation. The oxygen affinity of haemocyanin is not merely affected via the Bohr effect, but is also modulated by the concentration of organic ions such as L-lactate, urate and dopamine (Bridges & Morris 1986; Morris 1990). These organic effectors have the effect of increasing the oxygen affinity of haemocyanin.

The oxygen affinity of haemocyanin can also be affected by the haemolymph concentration of divalent cations such as Ca^{2+} and Mg^{2+} (Larimer & Riggs 1964; Truchot 1973, 1975; Mangum & Towle 1977; Mangum 1983; Morris *et al.* 1986). The formation of polymeric molecules from haemocyanin subunits occurs in crustacean haemolymph, and

the association into polymers requires the presence of either Ca^{2+} or Mg^{2+} ions. The degree of polymerisation of the haemocyanin subunits might affect their oxygen carrying capacity, and may affect the physical properties of the haemolymph, which in turn may affect its carrying capacity (Mangum 1983). Thus, alteration of the haemolymph calcium or magnesium concentration might affect the oxygen transporting properties of the haemolymph. Morris *et al.* (1988) concluded that the influence of salinity and oxygen stress on the intertidal prawn, *Palaemon elegans,* acted primarily via the alteration of haemolymph concentrations of calcium and magnesium. It is interesting to note that these and other authors have found a difference in the magnitude of the Bohr effect between animals acclimated to natural seawater and artificial seawater. These differences could not be attributed to a difference in the concentrations of calcium or magnesium between the two media. It is possible that a minor element in seawater might be responsible for the effect.

There also appear to be other modulators of haemocyanin affinity that have not been identified. These have been found as a result of the use of dialysed and non-dialysed haemolymph. The use of dialysed haemolymph resulted in lower oxygen affinities for haemocyanin in 11 species of crustacean (Bridges & Morris 1986). The haemolymph in these experiments was dialysed against a Ringers solution with L-lactate added. It now appears that some crustacean haemolymph contains a dialysable factor that reduces the oxygen affinity of haemocyanin (Wheatly & McMahon 1982b; Graham *et al.* 1983; Bridges *et al.* 1984; Bridges & Morris 1986; Morris 1990). The Ringer solutions used in these dialysis experiments may not have contained the correct concentrations of strontium or bromide since the solutions usually contain only the major ions found in the haemolymph (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}) (Lockwood 1961). Bromide is known to form charge transfer complexes with Cu^{2+} ions and thus may affect copper bearing enzymes and pigments. Strontium being a divalent cation might have a similar effect to Ca^{2+} and Mg^{2+}. Again this might explain the discrepancies in oxygen affinities of the haemocyanin of animals acclimated in natural and artificial seawater, and could also explain the discrepancies in the oxygen affinity of dialysed and non-dialysed haemolymph that have been attributed to an 'unidentified factor' of haemolymph.

The aim of this study was to attempt to establish the effect of salinity on the oxygen transporting properties of *Macrobrachium rosenbergii* haemocyanin. The effect of

varying salinity on ionic regulation has been studied in section 6.2, and when this information is combined with the data obtained from the following experiments a fuller picture of this prawns response to environmental salinity can be established.

The factors studied were the changes in: the oxygen carrying capacity of haemocyanin, the cooperativity of haemocyanin, and the pH of the haemolymph, in response to different environmental salinities. In addition, the PO_2 of pre- and post-branchial haemolymph was also determined for animals maintained in freshwater, and the post-branchial PO_2 of animals acclimated to 28°/ooS seawater was also determined.

8.2 Materials and methods

Adult *Macrobrachium rosenbergii* were exposed to 17.5 and 28.0 °/ooS seawater for seven days prior to sampling. The prawns were starved for two days prior to sampling. Eight intermoult prawns were used in each salinity exposure and an additional eight prawns were sampled from freshwater. These exposures were repeated to provide haemolymph for *in vivo* pH measurement. Thus six sets of eight prawns were sampled in total. The temperature at which the prawns were held was 28°C.

The method of salinity exposure, and haemolymph sampling is given in sections 3.3.2, 6.2.1. Serum was obtained from the haemolymph by the method described in section 3.3.3, and stored in a freezer (-20°C) in 'Eppendorf' tubes until required for analysis. Approximately 100-150µl of serum were obtained from each prawn. Prawns were not used more than once for this experiment.

8.2.1 *In vivo* haemolymph pH measurement

The *in vivo* pH of the prawns exposed to freshwater, 17.5 and 28 °/ooS seawater was measured using a semi-micro pH electrode (Russell pH, Auchtermuchty, Fife, Scotland) connected to a Corning model 255 pH meter. Haemolymph (≈ 350µl) was sampled from prawns and rapidly transferred to an 'Eppendorf' tube. The pH was measured

as quickly as possible to avoid changes due to CO_2 equilibration with the atmosphere.

8.2.2 *In vitro* oxygen dissociation measurement

In vitro oxygen dissociation for the haemolymph of *Macrobrachium rosenbergii* was determined spectrophotometrically using a diffusion chamber (Sick & Gersonde 1969, Zaimal et al. 1991). The changes in absorbance (at 335 nm) of haemocyanin that occur with changes in its degree of saturation were detected by means of two fibre optic cables connected to a spectrophotometer (Oriel Scientific 3090). A sample of haemolymph (3-5 µl) was pipetted onto a glass slide, which was then placed inside the diffusion chamber. The diffusion chamber was contained within a thermostatted water bath, which allowed the temperature of the chamber to be controlled. The haemolymph in the diffusion chamber was equilibrated with various O_2 and CO_2 mixtures, supplied by precision gas mixing pumps (Wosthoff M301). The PO_2 of the gas mixture was increased incrementally, while the PCO_2 was kept constant, and the change in absorbance at each PO_2 was recorded.

Dissociation curves were constructed at different pH's to examine the magnitude of the Bohr factor in *Macrobrachium rosenbergii.* The pH of the haemolymph was varied by altering the PCO_2 in the gas mixture, and the pH of the haemolymph was measured by tonometering another haemolymph sample (100µl), equilibrated with the same gas mixture, in a Radiometer BMS2. The pH at the P_{50} was determined by using the microcapillary pH electrode of the BMS2 connected to a digital pH meter (Corning model 255 ion analyser). The P_{50} of the haemolymph was estimated from the regression lines for the saturation values between 25% and 75% according to the Hill equation, and the n_{50} values were taken from the slopes of the regression lines.

The effect of temperature on the oxygen affinity of haemocyanin was investigated by constructing oxygen dissociation curves at three temperatures for each of the experimental salinities. The temperatures used were 22, 27 and 32°C, and these corresponded to temperatures within the environmental tolerance range for *Macrobrachium rosenbergii.* The effect of temperature on the oxygen affinity of haemocyanin can be quantified by calculating the change in enthalpy (ΔH) that

accompanies the oxygenation of haemocyanin, at constant pH (7.69), by the use of the following equation (Mangum 1983):

$$\Delta H = -2.303 \times R \times \frac{(T_1 \cdot T_2)(\Delta \log P_{50})}{(T_1 - T_2)} \quad (kJ.mol^{-1})$$

R = gas constant
T = absolute temperature (°K)

8.2.3 Haemolymph PO_2 measurement

The PO_2 of the pre- and post-branchial haemolymph was measured in intermoult adult prawns held in both freshwater and 28°/ooS seawater. Exposure to seawater, and sampling was as described in section 3.3.2, 6.2.1. Haemolymph PO_2 measurements were performed using an oxygen electrode (E5046, Radiometer, Copenhagen) which was contained within a thermostatted water jacket (at T°C) and connected to a small flow through sample cell. The electrode was connected to an oxygen meter (PHM72, Radiometer, Copenhagen). The oxygen electrode and meter were calibrated using air saturated deionised water, and a solution with a PO_2 of zero. The PO_2 of air saturated water was calculated according to the following equation:

$$PO_2 = (P_{atm} - WVP) \times 0.2095$$

PO_2 = Saturation partial pressure of O_2 (Torr) in freshwater at temperature T°C.
P_{atm} = Atmospheric pressure (Torr).
WVP = Water vapour pressure at temperature T°C (Torr).

A solution with a PO_2 of zero was freshly prepared for zero point calibration by the addition of a small amount of sodium sulphite to approximately $10cm^3$ of 0.01M sodium tetraborate solution.

Haemolymph was sampled from either, the ventral sinus (pre-branchial

haemolymph) or, from the pericardial sinus (post-branchial haemolymph). Prawns were caught and sampled as rapidly as possible to avoid alteration of the haemolymph PO_2. Care was taken to avoid disturbance of the prawns prior to capture to ensure that the PO_2 values obtained were from quiescent animals. After sampling the haemolymph was quickly injected into the sample chamber to avoid significant changes to the PO_2 .

The haemolymph of *Macrobrachium rosenbergii* was found to gel very quickly and this necessitated the dismantling and cleaning of the apparatus between each sample. The sample inlet lines and the chamber were washed with 'Tergazyme' between each sample, and then rinsed thoroughly with deionised water. This dismantling and cleaning required the recalibration of the equipment before another sample could be analysed. It was found that the saturated water calibration changed between samples, but the zero point calibration did not.

8.3 Results and discussion

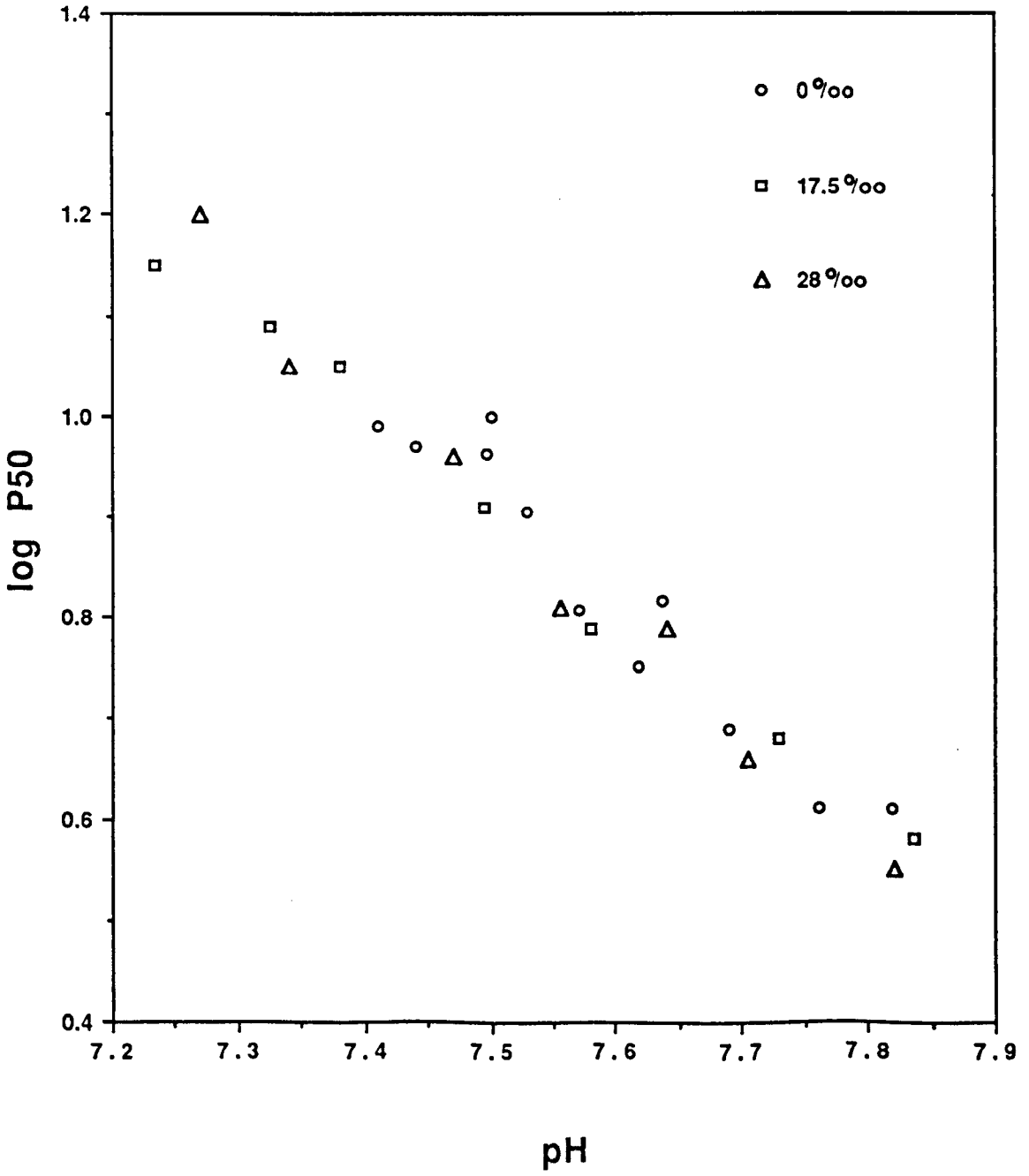
8.3.1 Effect of salinity on P_{50} (Figs.52,53,54,55)

The Bohr plots of P_{50} versus pH at three salinities show straight line relationships for the three temperatures analysed (Figs.52,53,54). The regression lines for the Bohr plots are given below in Table XXXIII. The slope of these regression lines is the Bohr factor, and is defined as:

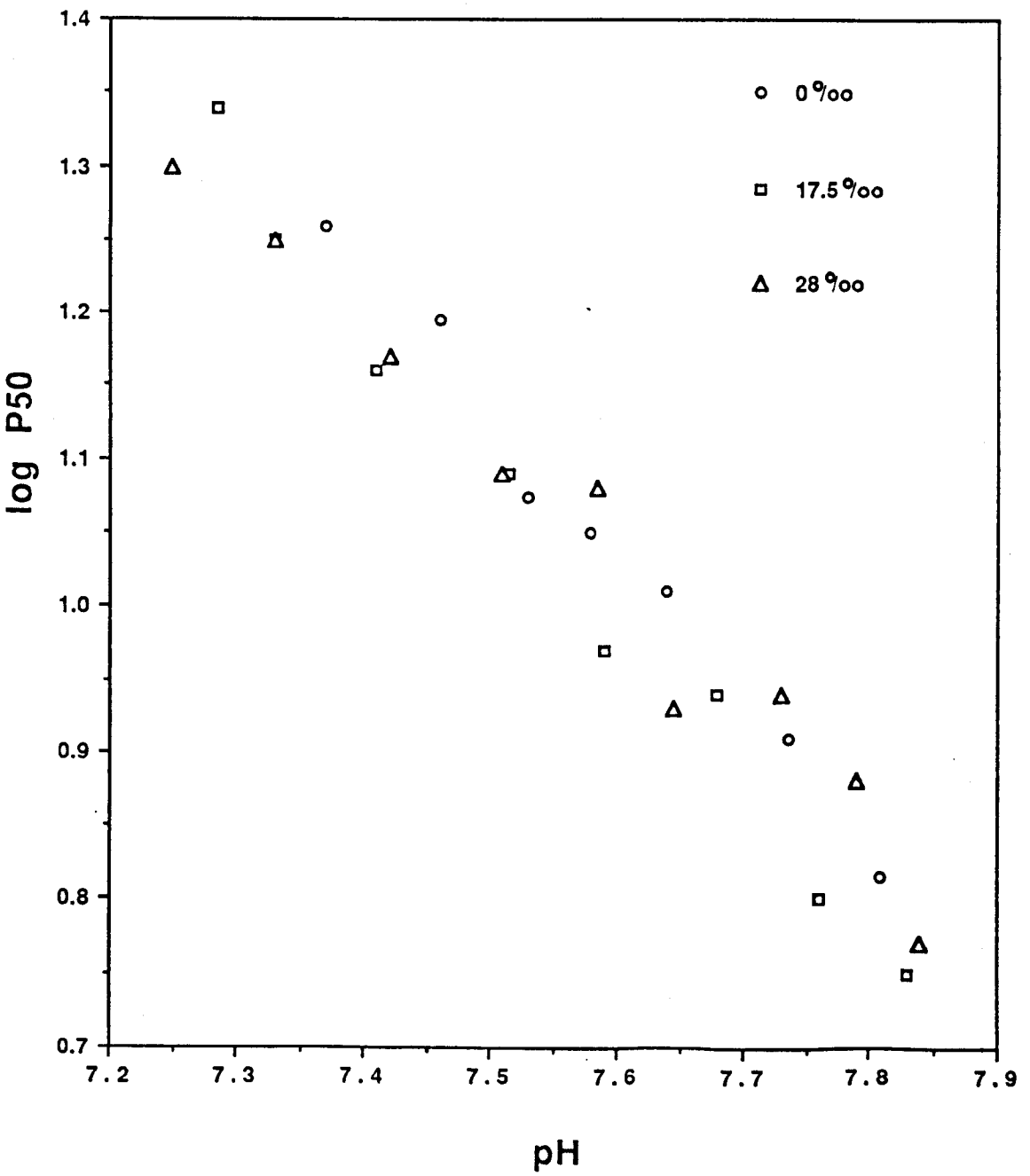
$$\text{Bohr factor} = \frac{\Delta \log P_{50}}{\Delta pH}$$

The Bohr values for the different salinities at each temperature were compared by covariance analysis. It was found that there was no significant effect of salinity on the Bohr values. The effect of temperature on the Bohr values was found to be significant.

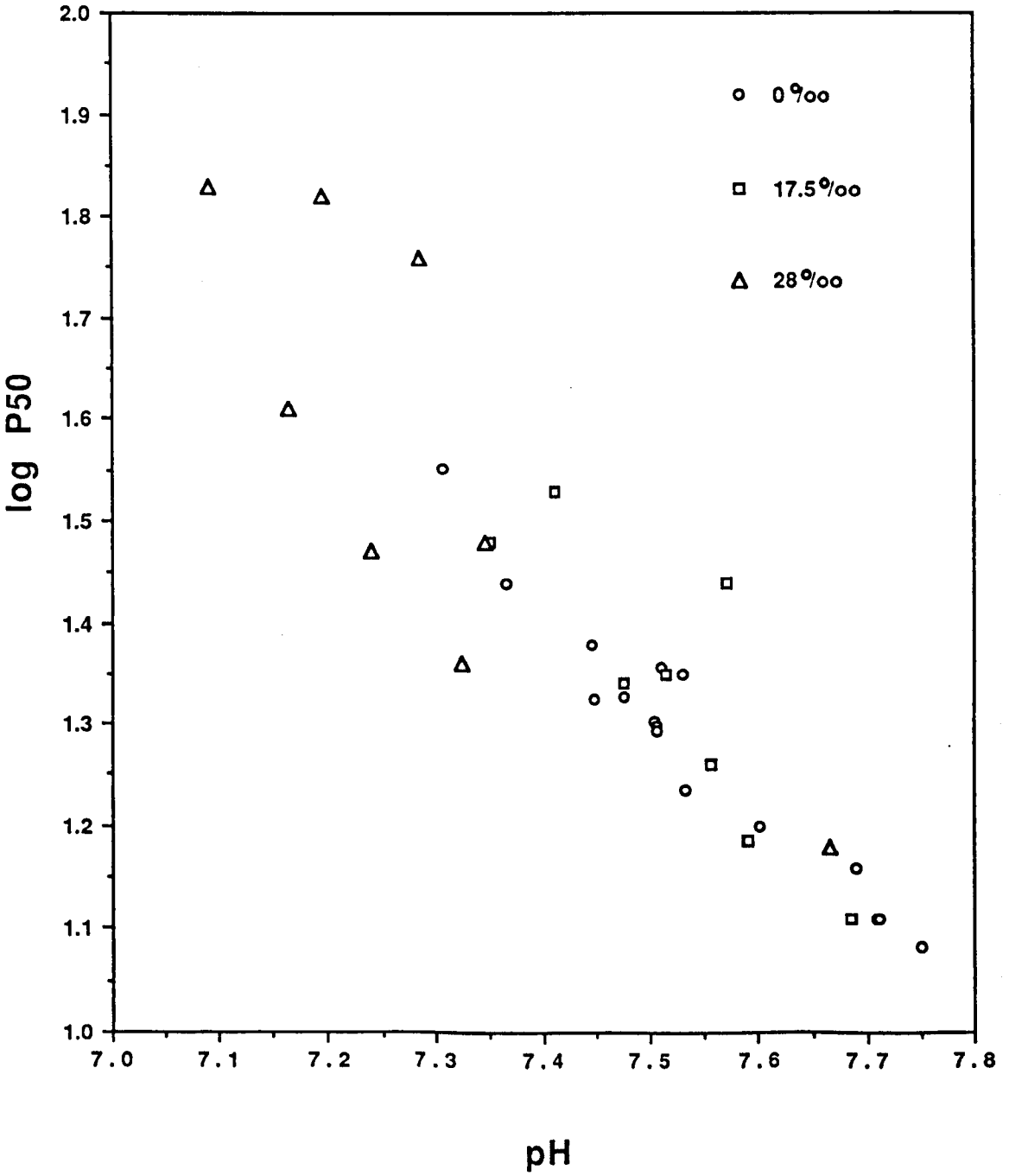
(Fig.52) Effect of pH on the P50 of haemocyanin from the freshwater prawn when exposed to different salinities, at 22 °c.



(Fig.53) Effect of pH on the P50 of haemocyanin from the freshwater prawn when exposed to different salinities, at 27 °c.



(Fig.54) Effect of pH on the P50 of haemocyanin from the freshwater prawn when exposed to different salinities, at 32 °c.



(Fig.55) Effect of temperature and salinity on the P50 of haemocyanin from the freshwater prawn.

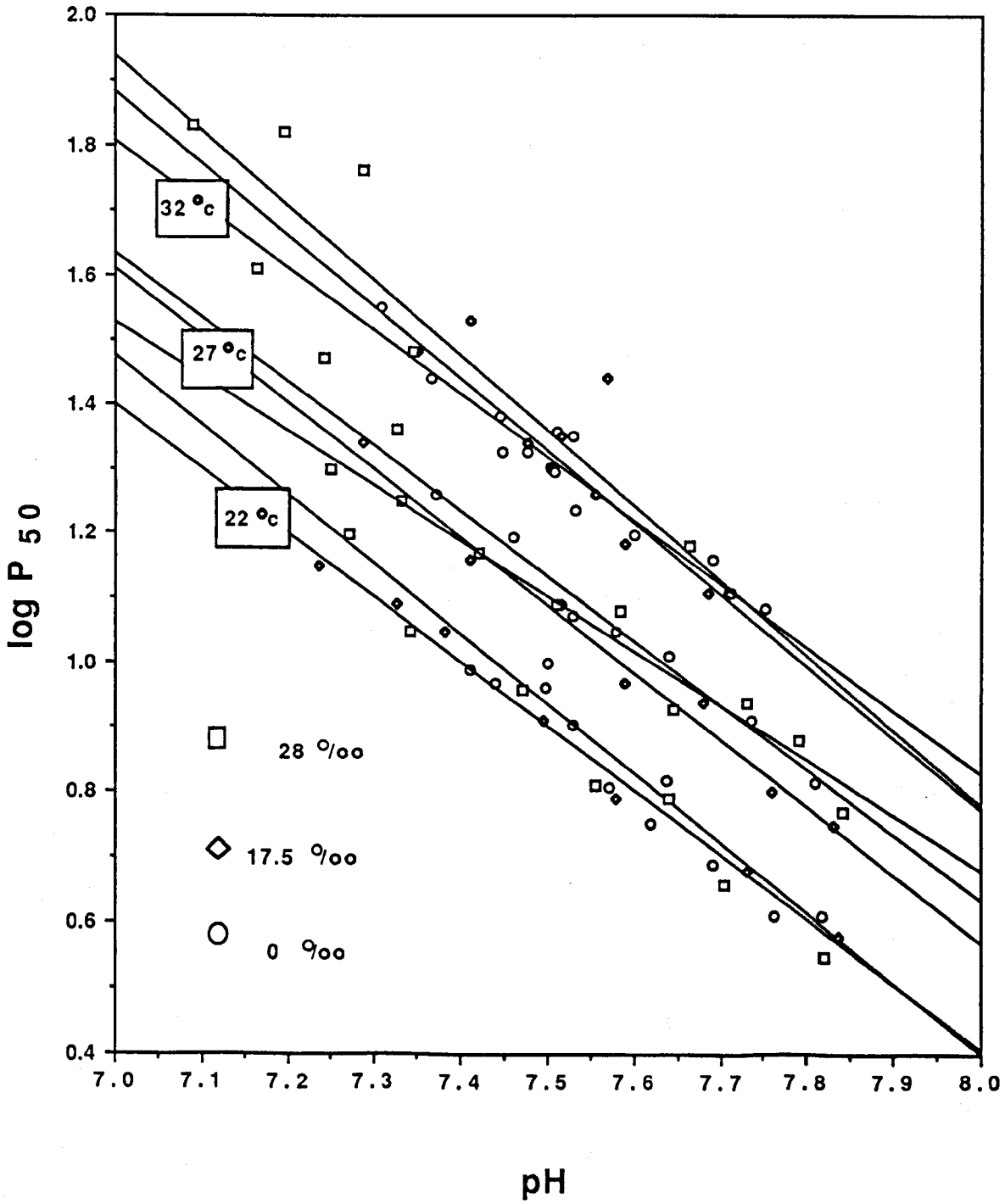


Table (XXXII) Regression analysis of Bohr plots of *Macrobrachium rosenbergii* haemocyanin at different temperatures and salinities.

salinity	temperature	regression equation	r
0 °/ooS			
	32°c	y = 8.69 - 0.980x	-0.968
	27°c	y = 8.62 - 0.998x	-0.994
	22°c	y = 9.14 - 1.090x	-0.960
17.5 °/ooS			
	32°c	y = 10.05 - 1.160x	-0.843
	27°c	y = 8.89 - 1.040x	-0.993
	22°c	y = 8.34 - 0.992x	-0.996
28 °/ooS			
	32°c	y = 9.65 - 1.110x	-0.830
	27°c	y = 7.47 - 0.850x	-0.983
	22°c	y = 9.39 - 1.130x	-0.990

When these regression lines are represented graphically at each temperature the effect of salinity on the P_{50} can be determined. From Figs.52,53,54 it is apparent that the three salinities tested do not cause a great variation in the P_{50} of *Macrobrachium rosenbergii* haemocyanin. The effect of temperature on the P_{50} of the haemocyanin is much greater (Fig.55). An increase in temperature causes an increase in the P_{50} of the haemocyanin. This can be seen as an adaptive feature as the solubility of oxygen in water decreases with an increase in temperature. The close relationship between the Bohr plots for different salinities deteriorates at the highest temperature tested (32°C). This temperature is approaching the upper thermal limit of approximately 35°C for *Macrobrachium rosenbergii* (New & Singholka 1985). The lower lethal temperature for this species is approximately 14°C (New & Singholka (1985) this is considerably below the lowest temperature (22°C) used in this experiment.

The Bohr factor for *Macrobrachium rosenbergii* haemocyanin does not

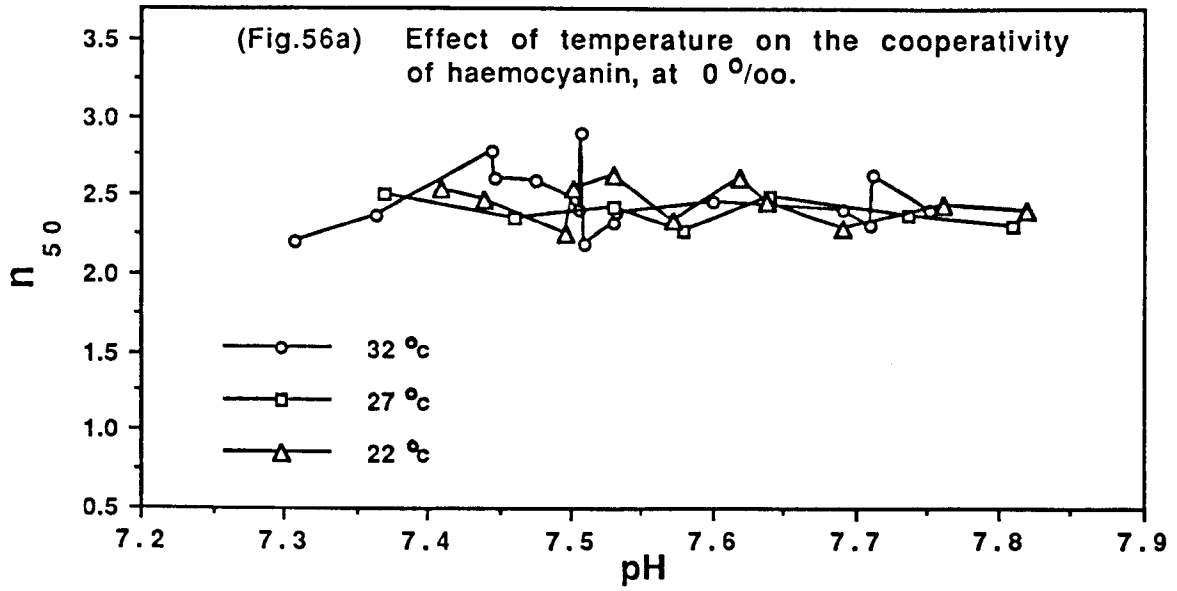
appear to vary greatly with either salinity or temperature, and has a range between -0.850 to -1.160. This range is suggested to be typical for marine crustaceans(-0.95 to -1.4) (Wheatly & McMahon 1982b), but is substantially higher than that found for the crayfish *Pacifastacus leniusculus*, *Procambarus clarkii* and *Orconectes rusticus* (-0.4 to -0.5). The lack of pH dependence of the P_{50} of *Pacifastacus leniusculus* haemocyanin is suggested to be an adaptation to life in freshwater (Wheatly & McMahon 1982b). If this is true it indicates that *Macrobrachium rosenbergii* has not adapted to life in freshwater in the same manner as the crayfish species. This is another indication that *Macrobrachium rosenbergii* is a recent coloniser of freshwater, and still retains many features of its palaemonid relatives.

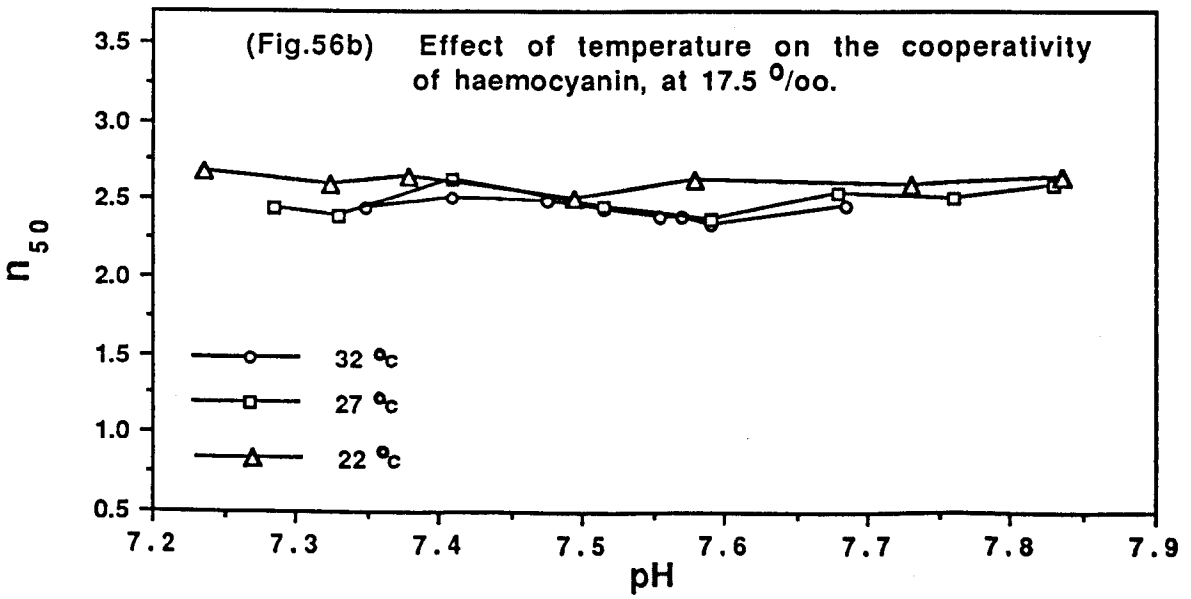
8.3.2 Cooperativity of haemocyanin (Figs.56a,b,c).

The degree of cooperativity of a respiratory pigment is the extent to which the binding of oxygen to that pigment is affected by oxygen molecules that are already bound. A cooperativity of 1 means that there is no effect of oxygen binding on the binding of subsequent oxygen molecules. A cooperativity above 1 causes the pigment to bind oxygen more strongly as additional oxygen molecules are bound. This effectively increases the amount of oxygen the respiratory pigment can carry.

The n_{50} plots for the cooperativity of the haemocyanin analysed in this work show that salinity has only a slight effect on the cooperativity of *Macrobrachium rosenbergii* haemocyanin (Figs.56a,b,c). Table XXXIIV gives the means and standard deviations for n_{50} at the three salinities and temperatures tested.

From Table XXXIV and Figs. 56a,b,c it can be seen that there is a slight increase in the n_{50} value with increasing salinity. This increase could be attributed to the slightly elevated calcium and magnesium concentrations found in the haemolymph at higher salinities. Overall, these n_{50} values lie in the middle of the range of values for crustaceans (≈2.0 - 4.5) given in the review by Mangum (1983).


(Fig.56a) Effect of temperature on the cooperativity of haemocyanin, at 0 °/oo.
n 50
3.5
3.0
2.5
2.0
1.5
1.0
0.5
32 °c
27 °c
22 °c
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
pH


(Fig.56b) Effect of temperature on the cooperativity of haemocyanin, at 17.5 °/oo.
n 50
3.5
3.0
2.5
2.0
1.5
1.0
0.5
32 °c
27 °c
22 °c
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
pH

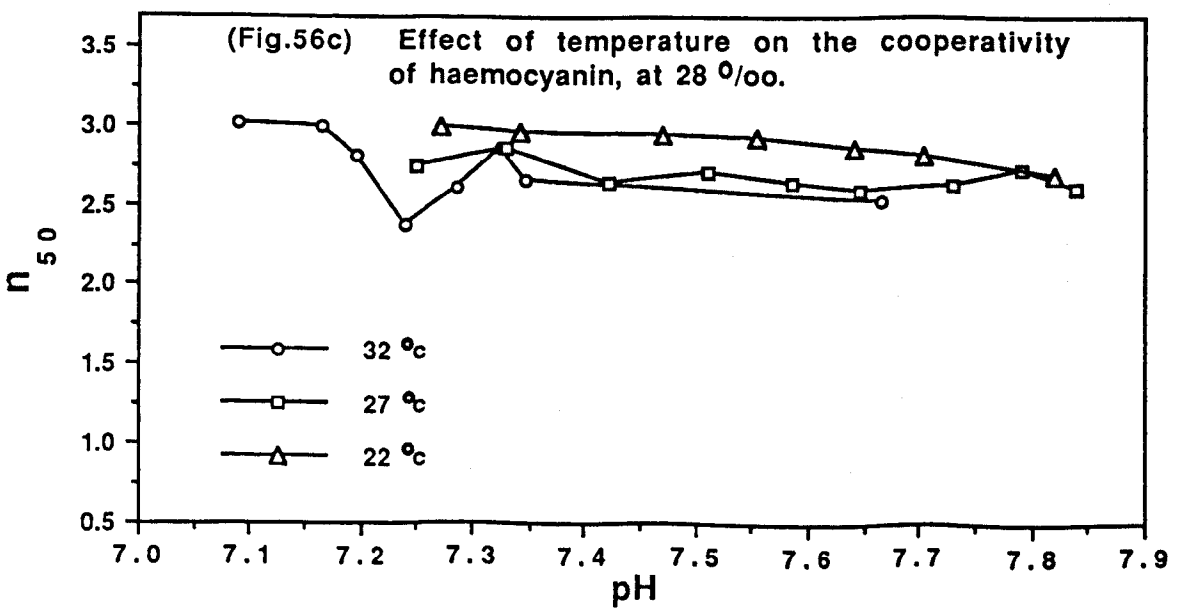

(Fig.56c) Effect of temperature on the cooperativity of haemocyanin, at 28 °/oo.
n 50
3.5
3.0
2.5
2.0
1.5
1.0
0.5
32 °c
27 °c
22 °c
7.0
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
pH

Table (XXXIV) Effect of temperature on n_{50} values for *Macrobrachium rosenbergii* haemocyanin from prawns exposed to different salinities. (Values as means ± s. d.).

Salinity (°/ooS)	Temperature (°C)	n_{50}
0	22	2.46 ± 0.12
	27	2.38 ± 0.09
	32	2.46 ± 0.19
17.5	22	2.61 ± 0.05
	27	2.49 ± 0.09
	32	2.42 ± 0.06
28	22	2.89 ± 0.10
	27	2.69 ± 0.10
	28	2.73 ± 0.21

The effect of temperature on haemocyanin cooperativity is unclear. At 17.5 and 28°/ooS the haemocyanin cooperativity decreases with increasing temperature. However, this relationship does not appear to exist in freshwater. It should be noted that the standard deviations of the n_{50} data are much greater for the freshwater adapted animals, and this could mask any effect of temperature.

There appears to be a slight decrease in cooperativity with increasing pH at 28°/ooS, although at 0 and 17.5°/ooS the cooperativity seems unaffected by pH.

8.3.3 Total oxygen carrying capacity of haemolymph.

The total haemolymph oxygen carrying capacity is calculated from the total haemolymph copper concentration (section 6.2.2.11), and assumes that all the haemolymph copper is incorporated into haemocyanin, and that two molecules of copper bind one of oxygen (Table XXXV). The linear relationship between haemolymph haemocyanin concentration and haemolymph oxygen carrying capacity was demonstrated in

Nephrops norvegicus by Hagerrman & Uglow (1985)

The oxygen carrying capacity of *Macrobrachium rosenbergii* haemolymph is quite high relative to the values for aquatic crabs (≈1-1.5) given in Mangum (1983). However, the values for *Macrobrachium rosenbergii* (3.8-2.1) are similar to those found for land crabs and for the shrimps *Crangon crangon* and *Callianassa californiensis.*

Table (XXXV) Haemolymph oxygen carrying capacity at different salinities, calculated from haemolymph copper concentration in *Macrobrachium rosenbergii.*

Salinity (^{o}/ooS)	[copper] (mmol.dm^{-3})	Haemolymph carrying capacity (cm^3 O_2/100 cm^3)
0.0	3.35	3.76
7.0	2.81	3.15
11.5	2.45	2.75
18.0	2.50	2.80
25.0	1.89	2.11
28.5	2.53	2.84

Mangum (1983) suggests that there may be a correlation between high haemocyanin concentrations and high hydrostatic pressures in the heart, due to colloid osmotic pressure, and suggests that this might be necessary for the production of a hyposmotic urine (Mangum 1986).

The oxygen carrying capacity shows a decrease from 3.76 cm^3 O_2/100 cm^3 haemolymph in freshwater adapted prawns, to 2.75 cm^3 O_2/100 cm^3 haemolymph in prawns acclimated to 11.5 ^{o}/ooS. As mentioned in section 6.2.2.11, this reduction in the carrying capacity of the haemolymph is disadvantageous since oxygen solubility decreases with increasing salinity. However, if the haemocyanin is behaving as an oxygen reserve, and the prawn is utilising oxygen dissolved in the plasma, then this reduction in the concentration of haemocyanin will only affect the prawns' ability to store oxgen for periods of burst swimming. The concentration of copper in the haemolymph, and hence the haemocyanin concentration is far greater in *Macrobrachium rosenbergii* than in many other species studied. Thus it might be suggested that the requirement for haemocyanin as an oxygen carrying pigment is secondary to its role in

maintaining the haemolymph osmotic pressure at low salinities. It was noticed that the colour of the haemolymph as it was sampled from the prawns varied according to the salinity to which the prawn was exposed. In freshwater adapted prawns the haemolymph was almost colourless, but at higher salinities (17.5, 28 °/oo) the haemolymph was blue. Thus the degree of oxygen saturation of the haemocyanin seems to vary according to environmental salinity. This may be taken as another indication that haemocyanin is present in excess of its requirement for respiration in the haemolymph of prawns in freshwater. This apparent increase in the saturation of the haemocyanin in increasing salinity is extremely interesting because the effect of salinity on the oxygen transport characteristics of haemocyanin seem to be minimal. The slight effect of salinity on haemocyanin function could be attributed to the fairly constant internal environment maintained by *Macrobrachium rosenbergii* when exposed to a range of salinities. Thus, whilst salinity seems to have little effect upon haemocyanin function with regard to cooperativity and oxygen affinity, the PO_2 of its haemolymph does appear to increase as salinity increases. The mechanism whereby this occurs is unclear.

8.3.4 *In vivo* pH (Table XXXVI).

The *in vivo* pH of prawns acclimated to 0, 17.5 and 28 °/ooS shows the high degree of control that *Macrobrachium rosenbergii* is able to exert over its internal pH. Between freshwater and 17.5 °/ooS the haemolymph pH varies by only 0.06 of a pH unit (no significant difference, two sample t-test, $p<0.05$). Even in 28°/ooS seawater the difference in haemolymph pH is only 0.19 of a pH unit (significant difference , two sample t-test, $p<0.05$).

Table (XXXVI) Haemolymph pH of prawns acclimated to two different freshwaters, and to two salinities.

salinity	0	0	17.5	28
medium pH	7.72	6.61	-	8.01
number	11	7	8	6
mean	7.700	7.685	7.640	7.511
s.d.	0.052	0.020	0.047	0.036

The slightly elevated pH of the haemolymph of prawns in freshwater, and the progressive acidosis with increasing salinity, is in agreement with the findings of Wheatly & McMahon (1982b) for *Pacifastacus leniusculus* (0 - 25.5°/ooS, pH = 7.95 - 7.81). This reduction in pH with hypersaline exposure might be due to reduced buffering of the haemolymph due to the reduced haemocyanin concentration found in the haemolymph at elevated salinities. The stable haemolymph pH found in salinities between 0-17.5 °/oo is another indication of this prawns' ability to maintain a stable internal environment in differing salinities. This is unsurprising in view of its recent estuarine history.

Table (XXXVII) Effect of salinity on haemolymph pH and P_{50} values of *Macrobrachium rosenbergii* haemocyanin.

salinity (°/ooS)	pH	log P_{50}	P_{50}
0	7.70	0.9354	8.62
17.5	7.64	0.9444	8.80
28	7.51	1.0857	12.18

The P_{50} values for *in vivo* pH of animals exposed to 0, 17.5 and 28°/ooS (Table XXXVII) show that there is little effect of salinity on the oxygen affinity of the prawns at salinities between 0-17.5 °/oo.

These P_{50} values at *in vivo* pH are at the lower end of the range given by Mangum (1983), but seem to be typical for marine and brackishwater decapods. At 28°/ooS there is an increase in the *in vivo* P_{50} value due to haemolymph acidosis. There is no adaptive advantage to this since it limits the tissues' ability to obtain oxygen from the haemocyanin via the Bohr effect. Again it should be mentioned that the haemolymph was a much darker blue colour at this high salinity, and thus the increased PO_2 may be a compensation for this elevated P_{50}.

The haemolymph pH values (7.70, 7.69) for the prawns held in freshwater at two different environmental pH's (6.6,7.7) show there is no significant difference (two sample t-test, $p<0.05$). The ability of this prawn to withstand acid conditions in

freshwater is quite surprising, since a pH of 6.6 is at the bottom of its environmental range. Indeed New & Singholka (1985) suggest a pH of 7.0 as a minimum for the culture of this species. The prawns exposed to this acid water survived and moulted without dying, however, there was an increased incidence of deformity and the prawns exhibited very poor pigmentation. This may have been due to prolonged compensation for the sub-optimal water pH. Animals maintained in the same manner, but in a pH of ≈7.0 did not manifest these symptoms.

8.3.5 Haemolymph PO_2.

The measurement of P_aO_2 and P_vO_2 of adult *Macrobrachium rosenbergii* in freshwater (Table XXXVIII) confirms the observations made in section 8.2.3 and 6.3.2.1. The mean pre-branchial P_vO_2 of 8.4 Torr is lower than most of the marine and brackishwater crabs (7-18 Torr) given in Table XXXII, but is similar to the values found for the crayfish *Pacifastacus leniusculus* (5 Torr) and *Astacus leptodactylus* (7 Torr).

Table (XXXVIII) P_aO_2 and P_vO_2 (Torr) values for *Macrobrachium rosenbergii* in freshwater. Animals quiescent.

Animal	P_aO_2	P_vO_2	difference
1	12.1	-	-
2	14.0	7.4	6.6
3	11.5	-	-
4	11.1	9.1	2.0
5	11.6	7.0	4.6
6	10.8	7.8	3.0
7	11.3	10.6	0.7
8	14.5	7.4	7.1
9	13.3	7.0	6.3
10	12.4	5.5	6.9
11	15.5	9.8	5.7
12	12.5	10.3	2.2
13	13.8	10.2	3.6
14	13.2	-	-
mean	12.7	8.4	
s.d	1.4	1.6	

The post-branchial P_aO_2 value of 12.7 is considerably lower than that found for the marine and brackishwater crabs (32-97 Torr), again this value is similar to that found for the crayfish. The low PO_2 of the post-branchial haemolymph is in agreement with the observation in section 8.2.3 regarding the pale colour of the haemolymph sampled from the pericardial sinus. However, the adaptive advantage of such a low oxygen concentration is obscure. It is possible that this is an adaptation to the freshwater environment. The small difference between the pre- and post-branchial PO_2 suggests that either there is a considerable barrier to oxgen diffusion, preventing saturation of the haemocyanin as it passes through the gills, or that the residence time of the haemolymph in the gills is too rapid to allow full oxygenation. The high haemocyanin concentration of the haemolymph and the low P_aO_2's measured means that the degree of oxygen saturation of the haemocyanin will be low; this explains the observation that the post-branchial haemolymph is almost colourless.

The low P_aO_2 values for *Macrobrachium rosenbergii* in freshwater cannot be explained by a salinity effect causing the loss of a factor from the haemolymph, and therefore affecting cooperativity or affinity. This is because there was no such effect on the cooperativity or affinity of prawns acclimated to 17.5 and 28 $^{o}/_{oo}S$.

Mangum (1983) comments that low PO_2 values recorded could be as a result of the respiration of microorganisms or protein residues from previous samples covering the electrode surface. This could not have occured in this experiment since the sample chamber was stored containing an enzyme detergent, and dismantled and washed with the enzyme detergent between each sample.

Effect of elevated salinity on P_aO_2.

The P_aO_2 of *Macrobrachium rosenbergii* haemolymph increases in when this prawn is acclimated to high salinity ($28^{o}/_{oo}$). The change in haemolymph PO_2 between freshwater and $28^{o}/_{oo}S$ seawater is 4.2 Torr (significant difference, two sample

t-test, $p<0.05$), and this represents an increase in the oxygen tension of 32.9% (Table XXXIX). The increased P_aO_2 of the haemolymph in high salinity confirms the observation that the haemolymph is darker blue from animals maintained at high salinity, and that this is due to an increase in the oxygen saturation of the haemocyanin.

Whilst there is an increase of 32.9% in the P_aO_2 of the haemolymph between freshwater and 28°/ooS, the oxygen tension in the haemolymph of prawns acclimated to high salinity is still very low when compared with the values presented in table XXXII. This is quite surprising since the oxygen carrying characteristics of *Macrobrachium rosenbergii* haemocyanin (P_{50}, n_{50}, pH and ΔH) seem to be similar to the other marine and brackishwater species so far studied. Thus, if the haemocyanin were saturated and in equilibrium with the plasma, the PO_2 of the haemolymph should be similar to those marine and brackishwater species. Since this is not the case it must be concluded that *Macrobrachium rosenbergii* haemocyanin is undersaturated, even at high salinity.

The increase in PO_2 of the haemolymph with increased salinity raises an important question; namely, how does this increase occur when the oxygen transporting characteristics of the haemocyanin of this species appear to be unaffected by salinity (sections 8.2.1, 8.2.2, 8.2.4)? A possible answer to this question is that the prawn increases the flow of haemolymph to the gills when exposed to high environmental salinity.

Table (XXXIX) P_aO_2 values for *Macrobrachium rosenbergii* in freshwater (27°C) and 28°/ooS seawater. Animals quiescent.

	Haemolymph P_aO_2 (Torr)	
	freshwater	28°/ooS seawater
number	14	8
mean	12.7	16.9
s.d	1.4	3.6

It has been suggested in the previous section that the haemocyanin of *Macrobrachium rosenbergii* is not fully saturated due to a lack of gill permeability, or lack of perfusion of the gills by the haemolymph. An increase in haemolymph perfusion of the prawns gills when exposed to high salinity would have two effects. Firstly it would allow greater saturation of the haemocyanin. This would also explain the elevated haemolymph PO_2 at high salinity. The elevated PO_2 would compensate for the slightly lower solubility of oxygen in the medium. However, post-larval and juvenile *Macrobrachium rosenbergii* do not increase their oxygen consumption when exposed to salinities between 0-28°/oo (Stephenson & Knight 1980; Nelson *et al.* 1977). Thus, the greater saturation of the haemocyanin at high salinity does not seem to be a response to an increased metabolic demand. Secondly, the chloride cells of the gills would have a greater volume of haemolymph passing them and this would allow an increased rate of excretion of chloride. This is necessary since the prawn maintains chloride hypo-ionic to the medium even at 28°/ooS (section 6.2.2.2). The counter-ion in this mechanism is thought to be bicarbonate (section 6.1.3, Fig.11) and thus the haemolymph bicarbonate should increase due to chloride excretion, this would cause an increase in the haemolymph pH. The converse actually occurs with a reduction in haemolymph pH at high salinities, this cannot be easily explained.

An alternative view of the apparent change in perfusion or permeability is that the prawn minimises the flow of haemolymph past the gills when it is in freshwater. The gills are the most permeable part of a crustacean and represent ≈90% of the external surface area of a crustacean (Cameron & Mangum 1983). Thus, when in freshwater, ionic loss across the gills could be unacceptably high if the prawn did not minimise the contact of the haemolymph with the external medium. The concept of a reduction in permeability as a mechanism involved in the adaptation to low salinity has been mentioned in section 6.0, although whether the permeability reduction is a physiological change, or a physical change in the composition of the crustacean integument, has not been explored. The reduction or change in the supply of haemolymph to the gills as a means of reducing ionic loss should be a comparatively easy physiological adaptation to make and as such is probably more likely to occur than a change in the composition of the integument.

CHAPTER 9 - Conclusions.

This work has sought to demonstrate some of the methods by which *Macrobrachium rosenbergii* has been able to establish itself in freshwater, and some of the constraints by which it is still linked to the sea. This has involved the study of this prawn at various stages of its lifecycle in order to reveal to what extent it has retained the physiological characteristics of the brackishwater palaemonids, and how it may have modified these to suit life in freshwater. Equally, comparisons can be drawn between how this species resembles other Crustacean groups that are more fully adapted to freshwater.

The first part of this study was concerned with why the larval stages of this species require brackishwater for development. The maintenance of a brackishwater larval phase by this species can be understood from the viewpoint of reproductive strategy. Those *Macrobrachium* species that are able to complete their lifecycles entirely in freshwater have had to sacrifice fecundity in order to do so. The lower fecundity of these species is due to the extended larval development within the egg, and the subsequent hatching of a well developed zoeal or postlarval stage. The zoeal stage of *Macrobrachium hendersonayanum* clings to the female before moulting to postlarva (Jalihal & Sankolli 1975), presumably to avoid being washed downstream. It was also noted that the zoeal stage in this species does not appear to be planktonic. The extended development that occurs within the egg in the exclusively freshwater species allows them to overcome three of the major problems that are raised by the transition between brackishwater and freshwater. These are:

1) The loss of a requirement for planktonic feed for small larvae.
2) The avoidance of osmo- and iono-regulatory stress during moulting
3) The avoidance of displacement from the adult habitat.

Thus the pressure upon a *Macrobrachium* species to abbreviate its larval development and adopt a more completely freshwater mode of existence seems to be connected to the ease with which adults can migrate back to brackishwater. This seems to be the case with *Macrobrachium nipponense*, in which populations from the upper reaches

of the river system are becoming less fecund, and producing larger eggs (Mashiko 1990). The larval environment must be considered as well. If a river system discharges into a mangrove, brackishwater lagoon or delta system, this would favour planktonic larval stages. This type of environment is also typified by quite slow moving water due to the plain like nature of the lower reaches of these rivers. It is this that gives rise to these geographical features. However, if a river is fast flowing, and empties into a relatively well scoured coastline this would not provide the type of nursery area that the small planktonic larvae require. Thus, different river systems will favour different reproductive strategies. The fact that the female palaemonid prawns carry their eggs on their pleopods rather than release them, as do the penaeid shrimp, is another indication as to how the Palaemonidae are adapted to estuarine life. By carrying the eggs until hatching, the zoeal larvae hatch in the estuarine environment, and are sufficiently large to be able to maintain themselves within this environment. This adaptation supports Panikkar's (1941) hypothesis that the Palaemonidae have reinvaded marine habitats from either brackish or freshwater.

It has been demonstrated that a relatively basic artificial seawater medium can support survival and growth in *Macrobrachium rosenbergii* larvae comparable to that achieved in natural seawater. It was only possible to demonstrate the essentiality of bromide addition in this artificial seawater. Addition of low concentrations of this ion to artificial seawater allowed good short term survival for the larvae. This suggested a possible uptake mechanism for bromide. Clarification of the lowest possible environmental concentration tolerated by the larvae will require longer trials, this would involve feeding the larvae which might confuse the results. Any other trace elements the larvae might require appear to be provided as impurities of the salts used in the artificial seawater, or be contained within the artemia fed to the larvae. The implication of this to a recirculating *Macrobrachium rosenbergii* hatchery are that trace element addition might not be necessary. Certainly once the biological filters are established, and food and faecal material is broken down, trace elements would be restored to the system. As Spotte (1979) mentions, recirculation systems are a sink for elements. The significant loss of the major ions is not likely, but the loss of bromide and strontium from the medium must be considered. This can be calculated from the bromine and strontium concentrations of stage X-XI larvae. Removal of these from the system after metamorphosis represents the loss of strontium and bromine from the medium. One litre of 12 $^{o}/_{oo}$ seawater provides enough strontium and bromine for 1016 and 28,283 stage X-XI larvae respectively. In an

intensive larval rearing system final stocking densities would range between 50 - 100 larvae.dm^{-3}, so an artificial seawater could supply the strontium required for 10-20 rearing cycles. This argument does not consider the losses of these ions that occur at every larval moult. It also does not consider the concept of a minimum threshold concentration for strontium. Thus it can be seen that there is a real possibility for depletion of strontium in recirculated water. Bromine seems unlikely to be significantly depleted, providing it is present at its seawater concentration, this is not guaranteed if some of the more basic artificial seawater formulations were used.

Since the basic artificial seawater devised in this study will support the development of larval *Macrobrachium rosenbergii* past metamorphosis, the feasibility of an inland recirculating hatchery for this species is increased. The use of crude salts will provide ample trace elements, and only the addition of strontium and bromide would be required. The other salts are commonly available in inland areas. Adequate chelation of the impurities present in the crude salts might be vital. Crude rocksalt often contains very high concentrations of iron, this can be largely sedimented out after strong aeration, but what remains should be chelated with EDTA.

Addition of excess bromide to the larval rearing medium did not adversely affect the larval growth and survival, so the range of concentrations tolerated by the larvae is quite wide. The apparent requirement by the larvae of *Macrobrachium rosenbergii* for bromide in their rearing medium suggests another reason why the larvae must spend their time of development in brackishwater. Seawater is rich in trace elements, far more so than freshwater, thus the method by which the prawns obtain these elements may differ between the two environments. In seawater, direct absorption from the medium may occur, this is unlikely in freshwater where these elements would probably be entirely derived from the food.

The brackishwater environment is a medium with an ionic concentration similar to that of the adult body fluids, (and those of the larvae, by implication). Whilst the ionic composition of brackishwater is rather different from that of the adult body fluids, it is still similar with respect to most of the major ions. If the larval ionic composition is similar to that of the adult, the pressures on the larvae in brackishwater to regulate their ionic composition are considerably reduced. This is not the case in freshwater, where there are both ionoregulatory and osmoregulatory pressures on the

larvae. A small larva with a high surface to volume ratio, and possibly underdeveloped powers of ionic regulation would be unable to resist the influx of water and loss of ions, paticularly if there were permeability changes associated with moulting.

Bromide seems to be implicated in the process of sclerotisation, and larval exuviae were found to contain ≈1000 ppm bromine. It is interesting that the pre-ecdysial procuticle (section 7.1) is sclerotised prior to moulting in Crustacea. It has been suggested that the reason for this was that this sclerotised cuticle resisted the action of the moulting fluid that digests the old cuticle for reabsorption (Stevenson 1983). It is possible that the sclerotised cuticle is a fundamental part of the cuticle barrier between the body fluids and the environment. This would explain the requirement for sclerotisation prior to moulting, since it would render the newly moulted prawn quite impermeable over most of its body surface. This would result in the animal only having to excrete the water taken in prior to moulting and would not result in a major ionic imbalance of the body fluids.

There is some evidence that this prawn is able to exert a high degree of regulation over its internal environment during the pre- and postmoult period presented in this study. The haemolymph magnesium, strontium and bromine concentrations are extremely well regulated during the pre- and postmoult period (section 6.3.2). This suggests that the concept of the prawn being extremely permeable during the moult might not be correct. What is more plausible is that the prawn becomes more permeable to water across the gills prior to moulting. This water is then excreted during the postmoult period. *Macrobrachium rosenbergii* is able to excrete a hypo-osmotic urine and thus re-establishment of the normal blood osmotic pressure would be relatively easy. The evidence for this increase in apparent permeability across the gill is the increased PO_2 of the haemolymph during the premoult period. This is assumed due to the dark blue colouration of the haemolymph during this period, and correlates with the higher PO_2 measurements of arterial haemolymph from prawns exposed to high salinity.

If one of the functions of scleroproteins in the cuticle is to reduce permeability, then the deliberate incorporation of halides into scleroproteins can be understood. The halogenation of scleroprotein might increase its water repellent characteristics. An alternative function is that it may confer greater elasticity to the

pre-ecdysial procuticle so that the expansion and stretching of the cuticle can occur. during and after moulting.

If bromide is essential to the adult prawns, their ability to obtain it from the freshwater medium will be limited, but dietary sources should contain enough to allow the prawn to extract it. It is interesting to note that Hedgpeth (1949) remarks that *Macrobrachium ohione* could be caught clinging to cottonwood twigs due to its habit of eating the leaves. Wright (1980) reported that enhanced recalcification occurred if *Gammarus pulex* was fed oak leaves after moulting. Studies upon phytophagous insects have revealed that plant polyphenols are incorporated into the integument during sclerotisation (Bernays & Woodhead 1982; Visser & Minks 1982). It was presumed by these authors that in conditions where diets might be low in dietary protein, especially the amino acid phenylalanine, the plant compounds could substitute for the aromatic compounds required for sclerotisation. The feeding of plant leaves in addition to an artificial diet improved growth and apparent health in pond cultured *Macrobrachium rosenbergii* (Harpaz & Schmalbach 1986). Maw & Kempton (1982) found that in peats, and soils with high organic contents, inorganic bromide accounted for only 8% of the total bromine present. The rest of the bromine was organically bound (total Br content ≈141ppm dry weight). They presumed this was in the form of brominated phenols and polyphenols which were of plant origin. These compounds are analogous to the compounds found in insect cuticles. A requirement for bromine in an organic form may be an additional reason why *Macrobrachium rosenbergii*, and other crustaceans consume their exuviae after moulting. The inclusion of brominated (or iodinated) phenols, polyphenols or amines, in artificial diets for crustaceans might improve growth, and/or possibly reduce adult and larval cannibalism. The ionic composition of inland salt lakes differs from that of seawater. Since bromine incorporation into sclerotised cuticles appears to be a function of environmental concentration, a low bromide concentration in an inland salt lake might be reflected in the crustacean species that inhabit this habitat. It is possible to speculate that this might be another reason why artemia from inland salt lakes differ in nutritional quality from those of a marine origin.

Whether there is a larval requirement for strontium was not demonstrated during this study, although preliminary investigations were attempted. The concentration of strontium in seawater is ten times lower than that of bromide, and thus it is more difficult to lower its concentration in a rearing medium. There is some evidence that the

adults have a requirement for strontium. This is provided by the discovery that the adult prawns regulate this ion in varying salinity. Additional evidence for a role for strontium was provided by the study of its concentration in the haemolymph during the moult cycle. A large peak in the haemolymph strontium concentration occurred just prior to moulting. The adult prawns in freshwater maintain a haemolymph concentration of strontium equivalent to the concentration found in brackishwater at ≈11-12°/oo. This concentration cannot be attributed to the inadvertent uptake of strontium during the calcium metabolism. This is especially the case when the regulation of this ion in varying salinity is considered. If its presence was a function of calcium metabolism it would not be as strongly regulated as it appears to be. The apparent relationship between strontium and magnesium concentrations in the prawns and larvae (sections 6.2.2.10,7.4.2.5) suggests that the regulation of these two ions may be linked, but the reason for this is unclear. It may be that magnesium is acting as a counter ion during strontium (re)absorption. It can be understood that a relationship should exist between these two ions over a narrow range of salinities, but for this relationship to be maintained in freshwater is indicative that the ratio between these ions is in some way important.

The peak in the haemolymph strontium concentration prior to ecdysis is suggestive of some role for this ion in the moulting process. There is some evidence that it may affect the calcification process (Bidwell *et al.* 1986,1990). A major site of strontium deposition is the cuticle and reabsorption of strontium from the old cuticle might be an important source of this ion to the prawn if it is required during the moulting process. Equally, as in the case for bromine, consumption of the exuviae may provide a means for retaining this ion.

The study of the osmotic and ionic regulation of adult *Macrobrachium rosenbergii* demonstrated the considerable degree of control it is able to exert over its internal osmotic and ionic environment. between 0-16°/oo *Macrobrachium rosenbergii* is able to regulate its haemolymph osmotic pressure, Na, Cl, Mg, Sr and K concentrations at approximately the level present when the prawn is in freshwater. This maintenance of a relatively stable internal environment between fresh and brackishwater suggests that *Macrobrachium rosenbergii* still retains the osmoregulatory features of a brackishwater palaemonid prawn, and has modified these to suit a life in freshwater.

The manner in which the adults regulated their haemolymph sodium and

chloride concentrations appear to be adaptive to life in freshwater. The evidence for this is the almost equimolar concentrations between the two ions in the haemolymph. This strategy seems to have been adopted by the Astacoid and Parastacoid crayfish in freshwater. The usual reason given for the dissimilarity in the haemolymph sodium and chloride concentrations is that the chloride is compensating for a high haemolymph magnesium concentration. Since the haemolymph magnesium concentration is so low in *Macrobrachium rosenbergii* it is reasonable to suppose that the chloride concentration should be reduced. However, the equimolar concentrations of sodium and chloride are maintained in brackishwater, even beyond the isosmotic point for sodium. The relationship between the sodium and chloride concentrations suggests that the two are taken up in equimolar concentrations, possibly via the gut. The gut seems a likely site for the absorption of sodium and chloride since the food of this prawn is far richer in these ions than the freshwater environment. If this is the case then it could be envisaged that the gills act as an excretory organ to maintain the ionic balance and their role in active uptake might be considerably reduced.

The apparent close link between the haemolymph osmotic pressure and the haemolymph sodium concentration suggests some sort of mechanism by which the prawn is able to control the osmolarity of its body fluids. It is well known that the free amino acid fluxes between the haemolymph and cellular environments preserves cell volume, but the mechanism by which the haemolymph osmolarity is controlled is not. Whilst it seems that sodium and chloride provide the bulk of the osmotic pressure of the haemolymph, and haemocyanin contributes via the effect of colloid osmotic pressure, it is not clear how this is controlled. If the osmotic pressure of the haemolymph and sodium concentration worked on a feedback mechanism this would enable the prawn to control its haemolymph osmotic pressure. In this case the control of chloride concentration would be such that it equalled that of sodium. The extremely high haemolymph haemocyanin concentration of this species relative to brackishwater crustaceans, is another indication as to the manner in which *Macrobrachium rosenbergii* has modified existing physiological mechanisms as it adapted to life in freshwater.

The fluxes in haemolymph haemocyanin concentration as a response to fluctuating environmental salinity are well known in brackishwater crustaceans (section 6.2.2.11). It appears that *Macrobrachium rosenbergii* has taken this process one step further by nearly trebling the concentration of haemocyanin in the haemolymph. This

would have the effect of increasing the osmotic pressure of the haemolymph. This high colloid osmotic pressure is intriguing since this species produces a very hyposmotic urine when in freshwater. Mangum (1986) suggests this might be due to increased hydrostatic pressures in the haemolymph caused by increased heart activity. The decrease in the perfusion of the gill that seems apparent when this species is in freshwater would reduce the pressure loss in the vascular system, and this might be the method by which the prawn maintains a large haemolymph hydrostatic pressure.

An anomaly of this increased haemolymph haemocyanin concentration is the low PO_2 of the haemolymph of prawns held in freshwater (section 8.3.5). Thus, whilst the prawns have a greater capacity for oxygen transport in the haemolymph than their brackishwater relatives, they do not appear to saturate the haemocyanin under normal conditions. I have suggested that this might be due to a reduction in the perfusion of the gill by haemolymph. During periods of osmoregulatory stress, such as the premoult stage or exposure to high environmental salinities, *Macrobrachium rosenbergii* appears to oxygenate its haemolymph to a greater degree. Whether this is to increase oxygen uptake for metabolic processes, or to allow greater ionic exchange across the gills is uncertain. It is possible that the greater perfusion of the gills during premoult allows a greater uptake of water to swell the prawn. This is in agreement with the earlier postulation that the permeability of the cuticle of the prawn does not change radically during the moult cycle, and it is merely increased water fluxes across the gills that give this impression.

The study of the oxygen transporting characteristics of the haemolymph (sections 8.3.1 - 8.3.4) revealed that salinity had little effect on haemocyanin. The cooperativity and P_{50} of *Macrobrachium rosenbergii* haemocyanin was relatively unchanged in prawns exposed to salinities between 0 - 28°/oo. This was largely attributed to the control of haemolymph magnesium and calcium concentrations by this prawn. Salinity exposure caused a slight acidosis of the haemolymph, and this would affect the *in vivo* P_{50}. The oxygen transporting characteristics of *Macrobrachium rosenbergii* haemocyanin are broadly the same as those marine and brackishwater crustaceans already studied. It was found that the P_{50}, n_{50} and Bohr effect were within the ranges found for these species. This suggests that *Macrobrachium rosenbergii* has not changed its mechanism of oxygen transport as it has adapted to freshwater.

The effect of temperature on the oxygen affinity of *Macrobrachium rosenbergii* haemocyanin was much greater. The prawn does increase the PO_2 of its haemolymph when exposed to high environmental salinity, this might be explained in part by the higher P_{50} of the haemocyanin, but whether this can account for all of the increase is uncertain. The increase in PO_2 of the haemolymph might be achieved by an increase of gill permeability or increased perfusion of the gill, the latter being the more likely event.

Macrobrachium rosenbergii is an ideal species of prawn for physiological studies because of its ability to survive equally well in freshwater and slightly brackish water. Its large size and frequent moulting enable the study of haemolymph related processes that require large haemolymph samples. Its ability to withstand freshwater will enable future work to be performed to confirm the roles of strontium and bromine in crustacean physiology. This has tremendous implications for our understanding of how these two elements might perform functions in vertebrate physiology. Since strontium has been linked with calcification, it is possible that it plays a role in other calcification processes. It is found in human bones, but largely as a function of environmental concentration, as in crustaceans this high concentration in calcified tissue might be obscuring its true physiological function. A dysfunction in the regulation of strontium, or inadequate environmental concentrations, might result in abnormal calcification.

Bromine has been implicated in the process of sclerotisation, and if it performs the role of a cofactor for an enzyme system, this suggests a possible method by which it acts in nervous systems. Some of the intermediate tyrosine derivatives of the sclerotisation process are catechol compounds. These are dopa and dopamine (Fig.38, section 7.2.1). These catechols can act as neurohormones in a number of nervous processes, and are the precursors for other neurohormones such as nor-adrenaline and adrenaline. If bromide/bromine functions in controlling either the formation, or the metabolism of these compounds, this might explain how bromide appears to have an effect on nervous systems.

Bibliography.

Adelung D., Bucholz F., Culik B. & Keck A. (1987)
Fluoride in tissues of krill *Euphausia superba* (Dana) and *Meganyctiphanes norvegica* (Sars) in relation to moult cycle. *Polar Biol.* **7**,(1),43-50.

Ahearn G. A. (1978)
Allosteric co-transport of NaCl and Calcium by the intestine of freshwater prawns. *J. Membrane Biology* **42**,(3), 281-300.

Andersen S. O. (1972)
*Acta Chem. Scand.***26**,3097

Andersen S. O. (1976)
Sclerotization in insect cuticle. In *Perspectives in Experimental Biology,* Vol 1 Zoology (Spencer-Davies ed.), Pergamon,187-190.

Andrews P. (1967)
Uber den Blutchemismus des Flußkrebses *Orconectes limosus* und seine Veranderung im Laufe des Jahres. *Z. Vgl. Physiol.* **57**,7-43.

Anger K. & Nair K. K. C. (1979)
Laboratory experiments on the larval development of *Hyas araneus* (Decapoda, Majidae). *Helgolander wiss. Meeres.* **34**, 287-311.

Appelberg M. (1985)
Changes in haemolymph ion concentrations of *Astacus astacus* (L.) and *Pacifastacus leniusculus* (Dana) after exposure to low pH and aluminium. *Hydrobiologia.***121**,12-25.

AQUACOP (1977)
Macrobrachium rosenbergii (de Man) culture in Polynesia. Progress in developing a mass intensive rearing technique in clearwater. *Proc. World Maric. Soc.* **8**,311-326.

Armstrong D. A., Chippendale D., Knight A. W. & Colt J.E. (1978)
Interaction of ionised and unionised ammonia on short term survival and growth of prawn larvae *Macrobrachium rosenbergii. Biol. Bull.* **154**,15-31.

Armstrong D. A., Stephenson M. J. & Knight A.W. (1976)
Acute toxicity of nitrite to larvae of the giant Malaysian prawn *Macrobrachium rosenbergii. Aquaculture.* **9**,39-46.

Barnes H. (1954)
Some tables for the ionic composition of seawater. *J. Exp. Biol.* **31**,582-588.

Barnes R. D. (1980)
Invertebrate Zoology. 4thEdn. Holt-Saunders, Philadelphia.1089p.

Baumberger J. P. & Olmstead J. M. D. (1928)
Changes in the osmotic pressure and water content of crabs during the moult cycle. *Physiol Zool.***1**,(4), 531-543.

Bernays E. A. & Woodhead S. (1982)
Incorporation of dietary phenols into the cuticle in the tree locust *Anacridium melanorhodon*. *J. Insect Physiol.* **28**, (7), 601-606.

Bidwell J. P., Kuzirian A., Jones G., Nadeau L. & Garland L. (1990)
The effect of strontium on embryonic calcification of *Aplysia californica*. *Biol. Bull.* **178**,231-238.

Bidwell J. P., Paige J. A., & Kuzirian A. M. (1986)
Effects of strontium on the embryonic development of *Aplysia californica*. *Biol. Bull.* **170**,75-90.

Bidwell J. P. & Spotte S. (1985)
Artificial Seawaters. Jones and Bartlett. Boston. USA. 349pp.

Bjerregaard P. & Vislie T. (1986)
The effect of copper on ion and osmoregulation in the shore crab *Carcinus maenas*. *Marine Biology* **9**, 69-76.

Bookhout C. G. & Costlow J. D. (1970)
Nutritional effects of artemia from different locations on larval development of crabs. *Helgolander Wiss. Meeres.* **20**,435-442.

Boone R. J. & Manthey M. (1983)
The anatomical distribution of fluoride in various body segments and organs of Antatctic krill, *Euphausia superba* (Dana). *Arch. Fischereiwiss.* **34**,(1),81-85.

Boschi E. E. (1961)
Actas Trab. 1er Congr. Sud. Zool.,**2**, 69-77.

Bowen H. J. M. (1966)
Trace elements in biochemistry. Academic Press, London.

Bower C. E., Turner D. T. & Spotte S. (1981)
pH maintenance in closed seawater culture systems: limitations of calcareous filtrants. *Aquaculture.* **23**,(1-4), 211-217.

Brannon A. C. & Rao K. R. (1979)
Barium, strontium and calcium levels in the exoskeleton, hepatopancreas and abdominal muscle of the grass shrimp, *Palaemonetes pugio*: Relation to moulting and exposure to barite. *Comp. Biochem. Physiol.* **Vol 63a**,261-274.

Brick R. W. (1974)
Effects of water quality, antibiotics, phytoplankton and food on survival and development of larvae of *Scylla serrata* (Crustacea: Portunidae). *Aquaculture.* **3**, 231-244.

Bridges C. R. & Morris S. (1986)
Modulation of haemocyanin oxygen affinity by L-lactate - A role for other cofactors. In: *Invertebrate Oxygen Carriers.* (Linzen B. ed.), Springer, Berlin, 341-352.

Bridges C. R., Morris S. & Grieshaber M. K. (1984)
Modulation of haemocyanin oxygen affinity in the intertidal prawn *Palaemon elegans* (Rathke). *Resp. Physiol.* **57**,189-200.

Briggs M. R. P., Jauncey K. & Brown J. H. (1988)
The cholesterol and lecithin requirements of juvenile prawn (*Macrobrachium rosenbergii*) fed semi-purified diets. *Aquaculture.* **70**,121-129.

Broad A. C. (1957)
The relationship between and diet and larval development of *Palaemonetes. Biol Bull.* **112**,162-170.

Bryan G. W. (1967)
Zinc regulation in the freshwater crayfish (including some comparative copper analyses). *J. Exp. Biol.* **46**,281-296.

Cameron J. N. (1978)
Effects of hypercapnia on blood acid-base status, NaCl fluxes, and trans-gill potential in freshwater blue-crabs, *Callinectes sapidus. J. Comp. Physiol.* B. **123**,137-141

Cameron J. N. (1985a)
Post-moult calcification in the blue crab (*Callinectes sapidus*): relationships between apparent net H^+ excretion, Ca^{2+} and HCO_3^-. *J. Exp. Biol.* **119**,275-285.

Cameron J. N. (1985b)
Compensation of hypercapnic acidosis in the aquatic blue crab, *Callinectes sapidus*: The predominance of external seawater over carapace carbonate as the proton sink. *J. Exp. Biol.* **114**,197-206.

Cameron J. N. & Mangum C. P. (1983)
Environmental adaptations of the respiratory system: Ventilation, Circulation and Oxygen transport. In: *the Biology of Crustacea* (Vernberg F. J. and Vernberg W. B.) ch. 2,Vol 8,43-63, Academic press.

Campbell P. J. & Jones M. B. (1989)
Ionic regulation of the estuarine prawn *Palaemon longirostris,* Caridea, Palaemonidae. *Ophelia,* **30**,(2),141-154.

Canagaratnam P. (1959)
Growth of fishes in different salinities. *J. Fish. Res. Board Canada.***16**,121-130.

Castille F. L. & Lawrence A. L. (1981)
The effect of salinity on the osmotic sodium and chloride concentrations in the haemolymph of the freshwater shrimpd *Macrobrachium ohione* (Smith) and *Macrobrachium rosenbergii* (de Man). *Comp. Biochem. Physiol.* **Vol.70a**, (1), 47-53.

Cawthorne D. F., Beard T., Davenport J. & Wickins J. F. (1983)
Responses of juvenile *Penaeus monodon* Fabricius to natural and artificial seawaters of low salinity. *Aquaculture* **32**, 165-174.

Choudhury P. C. (1971)
Complete larval development of the palaemonid shrimp *Macrobrachium carcinus* (L.) reared in the laboratory. *Crustaceana.* **20**,51-69.

Christians O. & Leineman M. (1980)
Untersuchen uber fluor im krill (*Euphausia superba*). *Inf. Fischwirtschaft.* **6**,254-260.

Christians O. & Leineman M. (1983)
Uber die Fluoridwanderung aus den Schalen in das Muskelfleisch bei gefriergelalagertem antarktischem Krill (*Euphausia superba* Dana) in Abhangigkeit von der Lagertperatur und -zeit. *Arch. Fischereiwiss.* **34**,(1),87-95.

Culkin F. (1965)
The major constituents of seawater. In *Chemical Oceanography, Vol 1* (Riley J.P. and Skirrow G. eds.), Academic Press, London.

Dall W. (1965)
Studies on the physiology of a shrimp:-*Metapenaeus spp.* iii. Composition and structure of the integument. *Aust. J. Mar. Freshw. Res.* **16**,13-23.

Deaton L. E. (1981)
Ion regulation in freshwater and bivalve mollusks. *Physiol. Zool.* **54**, 109-121.

Deaton L. E. & Greenberg. M. J. (1986)
There is no horohalinicum. *Estuaries.* **9**,(1),20-30.

Denne L. B. (1968)
Some aspects of osmotic and ionic regulation in the prawns *Macrobrachium australiense* (Holthuis 1950) and *Macrobrachium equidens* (Dana). *Comp. Biochem. Physiol.* **26**,17-30.

Dennell R. (1960)
Integument and exoskeleton. In: *Physiology of the Crustacea.* (Waterman T. H. ed.) Vol. 1, Academic Press. pp.449.

Djangmah J. S. & Grove D. J. (1970)
Blood and hepatopancreas copper in *Crangon vulgaris* (Fabr.). *Comp. biochem. Physiol.* **32**,733-745.

Dodd J. R. (1967)
Magnesium and strontium in calcereous skeletons: a review. *J. Paleontol.* **41**,(6),1313-1329.

Doughtie D. G. & Rao K. R. (1982)
Rosette glands in the gills of the grass shrimp *Palaemonetes pugio.* I. Comparative morphology, cyclical activity and innervation. *Journal of Morphology.* **171**,41-67.

Dugan C. C., Hagood R. W. & Frakes T. A. (1975)
Development of spawning and mass larval rearing techniques for brackish-freshwater shrimps of the genus *Macrobrachium* (Palaemonidae). *Florida Marine Research Publications* **No. 12,** Florida Department of Natural Resources Marine Research Laboratory.

Dunson W. A. (1984)
Permeability of the integument of the Horseshoe crab, *Limulus polyphemus,* to water, sodium and bromide. *Journal of Experimental Zoology.* **230,** 495-499.

Eloffson R., Myhrberg H., Aramant R., Lindvall O. & Falk B. (1978)
Catecholaminergic salivary glands in *Gammarus pulex.* An electron microscopic and microspectrofluorimetric study. *J. Ultrastruct. Res.* **64**,14-22.

Felder D. L. (1978)
Osmotic and ionic regulation in several western Atlantic Callianassidae (Crustacea, Decapoda, Thalassinidea). *Biol. Bull.* **154**,409-429.

Fieber L. A. & Lutz P. L. (1982)
Calcium requirements for moulting in *Macrobrachium rosenbergii* (deMan). *J. World Maric. Soc.* **13**,21-27.

Fieber L. A. & Lutz P. L. (1985)
Magnesium and calcium metabolism during moulting in the freshwater prawn *Macrobrachium rosenbergii. Can. J. Zool.* **63**,(5),1120-1124.

Foskett J. K. (1971)
Osmoregulation in the larvae and adults of the grapsid crab *Sesarma reticulatum* (Say). *Biol. Bull.* **153**,505-526.

Frankel S. & Jellinek C. (1927b)
Ueber *Limulus polyphemus. Biochem. Z.* **185**,384.

Friedlander P. (1909)
Ber. deut. Chem. Ges. **42**,765-770.

Fujimura T. & Okamoto H. (1970)
Notes on progress made in developing a mass culturing technique for *Macrobrachium rosenbergii* in Hawaii. *Indo-Pac. Fish. Counc. Proc.* 14th. Sess., 1-18.

Fujita S., Watanabe T. & Kitajima C. (1980)
Nutritional quality of Artemia from different localities as a living feed for marine fish from the viewpoint of essential fatty acids. In: *The Brine Shrimp Artemia.* (Persoone L. G., Sorgeloos P., Roels O. and Jaspers E. eds.) **Vol. 3,** 277-290. Wettesen, Belgium, Universal Press.

George M. J. (1969)
Genus *Macrobrachium* (Bate 1868). *Bull. Central. Mar. Fish. Res. Inst.* **14**,178-216.

Gerard J. F. & Gilles R. (1972)
The free amino-acid pool in *Callinectes sapidus* (Rathbun) tissues and its role in the osmotic intracellular regulation. *J. Exp. Mar. Biol. Ecol.* **10**, 125-136.

Ghidalia W. (1985)
Structural and biological aspects of pigments. In *The Biology of Crustacea,* Vol 9 (Bliss D. and Mantel L. eds.),301-394, Academic Press.

Gibbs P. E. & Bryan G. W. (1972)
A study on strontium, magnesium and calcium in the environment and exoskeleton of decapod crustaceans, with special reference to *Uca burgersii* on Barbuda, West Indies. *J. Exp. Mar. Biol. Ecol.* **9**,97-110.

Goldberg E. D. (1965)
Minor elements in seawater. In *Chemical Oceanography, Vol 1* (Riley J.P. and Skirrow G. eds.), Academic Press, London.

Gomez-Diaz G. & Ohno A. (1986)
Possible significance of rearing conditions of ovigerous *Macrobrachium rosenbergii* (deMan). In *The First Asian Fisheries Forum, Asian Fisheries Society,Iloilo, Philippines.* (Maclean J.L., Dizon L.B. and Hosillos L.V. eds.).45-48.

Gomez-Diaz G. & Kasahara S. (1987)
The morphological development of *Macrobrachium rosenbergii* (de Man) larvae. *J. Fac. Appl. Biol. Sci.* **26**,(1-2),43-56.

Gomez-Diaz G. (1987)
Effect of environmental embryonic temperature on larval development of *Macrobrachium rosenbergii. J. Exp. Mar. Biol. Ecol.* **114**,(1),39-47.

Goodwin T. W. (1971)
Pigments - Arthropoda. In *Chemical Zoology* (Florkin M. and Scheer B.T. eds.) Vol VI, part B, Arthropoda, Academic Press.

Gordon H. T. & Welsh J. H. (1948)
The role of ions in axon surface reaction to toxic inorganic compounds. *J. Cell. Comp. Physiol.* **31**,395-415.

Goy J. W. & Costlow J. D. (1980)
Nutritional effects of *Artemia* from different geographical strains on larval development of decapod crustaceans. *Am. Zool.* **20**,888.

Graham G. A., Mangum C. P., Terwilliger R. C. & Terwilliger N. B. (1983)
The effect of organic acids on oxygen binding of haemocyanin from the crab *Cancer magister. Comp. Biochem. Physiol.* **74a**,(1),45-50.

Greenaway P. (1972)
Ca^{2+} regulation in the freshwater crayfish *Austropotamobius pallipes* (Lereboullet). *J. Exp. Biol.* **57**,471-487.

Greenaway P. (1974)
Calcium balance of the premoult stage of the freshwater crayfish *Austropotamobius pallipes* (Lereboullet). *J. Exp. Biol.* **61**,27-34.

Greenaway P. (1976)
The regulation of haemolymph calcium concentration of the crab *Carcinus maenas. J. Exp. Biol.* **64**,149-157.

Greenaway P. (1985)
Calcium balance and moulting in the crustacea. *Biol. Rev.* **60**, 425-454.

Greenaway P. & Lawson B. (1982)
Sodium balance in the australian crayfish *Cherax destructor, Eusastacus keirensis* and *Euastacus spinifer* (Decapoda, Parastacoidea). *Aust. J. Freshw. Res.* **33**,(3),507-515.

Greenaway P. & MacMillen R. E. (1978)
Salt and water balance in the terrestrial phase of the inland crab *Holthuisana (Austrotelphusa) transversa* Martens (Paratelphusoidea: Sundatelphusidae). *Physiol Zool.* **51**, 217-229.

Guttman R. (1939)
Stabilisation of spider crab nerve membranes by alkaline earths, as manifested by resting potential measurement. *J. Gen. Physiol.* **23**, 343-364.

Hackman R. H. (1964)
Chemistry of the insect cuticle. In *The Physiology of Insecta* (Rockstein M. ed.) ch.8,471-506, Academic Press, New York.

Hackman R. H. & Goldberg M. (1968)
A study of a melanic mutant of the blowfly *Lucilia cuprina. J. Insect Physiol.* **14**, 765-775.

Hackman R. H. (1971)
The integument of Arthropoda. In *Chemical Zoology* (Florkin M. and Scheer B.T. eds.) Ch. 1,Vol VI, part B, Arthropoda, Academic Press.

Hagerman L. (1973)
Ionic regulation in *Crangon vulgaris* (Fabr.) (Crustacea, Natantia) from brackish water. *Ophelia.* **12**,(1-2),151-157.

Hagerman L. (1980)
The regulation of Ca^{2+} and Mg^{2+} in the urine during the moulting cycle of *Crangon vulgaris* (Fabr.). *J. Exp. Biol.* **46**,51-88.

Hagerman L. & Uglow R. F. (1981)
Ventilatory behaviour and chloride regulation in relation to oxygen tension in the shrimp *Palaemon adspersus* Rathke maintained in hypotonic medium. *Ophelia.* **20**,(2),193-200.

Hagerman L. & Uglow R. F. (1982)
Effects of hypoxia on osmotic and ionic regulation in the brown shrimp *Crangon crangon* (L.) from brackish water. *J. Exp. Mar. Biol. Ecol.* **63**,93-104.

Hagerman L. & Uglow R. F. (1983)
The influence of temperature on the osmoregulation of the brackishwater shrimp *Palaemonetes varians. Ophelia* **22**,(2),229-236.

Hagerman L. & Uglow R. F. (1985)
Effects of hypoxia on the respiratory and circulatory regulation of *Nephrops norvegicus. Mar. biol.* **87**,273-278.

Harpaz S. & Schmalbach E. A. (1986)
Improved growth and health of the Malaysian prawn, *Macrobrachium rosenbergii*, by addition of fresh leaves to the artificial diet. *Aquaculture,* **55**, 81-85.

Harrison K. E., Lutz P. L. & Farmer L. (1981)
The ontogeny of osmoregulatiory ability of *Macrobrachium rosenbergii. Am.Zool.* **21**,(4), 1014.

Hedgpeth J. W. (1947)
River shrimps. *Prog. Fish Cult.* October,181-184.

Hedgpeth J. W. (1949)
The North American species of *Macrobrachium* (river shrimp). *Tex. J. Sci.* **1**, (3), 28-38.

L'Helias C. (1971)
Chemical aspects of growth and development in insects. In *Chemical Zoology* (Florkin M. and Scheer B.T. eds.) ch.12,Vol V, part B, Arthropoda, Academic Press.

Hempel G. & Manthey M. (1981)
On the fluoride content of larval krill *Euphausia superba. Meeresforsch/Rep. Mar. Res.* **29**,(1),60-63.

Henry R. P., Kormanik G. A., Smatresk N. J. & Cameron J. N. (1981)
The role of $CaCO_3$ as a source of HCO_3^- for the buffering of hypercapnic acidosis in aquatic and terrestrial decapod crustaceans. *J. Exp. Biol.* **94**,269-274.

Hernandez S. R de I. & Taylor A. C. (1985)
The effect of temperature on osmotic and ionic regulation in the prawn *Palaemon elegans* (Rathke). *Ophelia.* **24**,(1),1-15.

Holthuis L. B. (1949)
On some species of *Macrobrachium* from West Africa. *Eos.* **25**,3-4,175-185.

Holthuis L. B. (1950a)
Results of the Archbold expeditions No.63. the Crustacea, decapoda, Macrura collected by the Archbold New Guinea expeditions. *Amer. Mus. Novitates.* **Vol 1461,1-17.**

Holthuis L.B. (1950b)
Preliminary descriptions of twelve new species of palaemonid prawns from American waters. (Crustacea, Decapoda). *Proc. K. Akad. Wetenschap.* **53,1,93-99.**

Holthuis L. B. (1980)
FAO Fisheries Synopsis No.125. Vol1. Shrimps and Prawns of the World. F.A.O. Rome.(FIR/S125 Vol1).

Hsieh C. H., Chao N. H., Gomes L. A. de O. & Liao I. C (1989)
Culture practices and status of the Giant freshwater prawn, *Macrobrachium rosenbergii* in Taiwan. Paper presented in the Third Brazilian Shrimp Farming Congress, 15-20 October, Joao Pessoa- P.B., Brazil. 25pp. (unpubl. ms.).

Hughes D. A. & Richard J. D. (1973)
Some current directed movements of *Macrobrachium acanthurus* (Wiegmann 1836) under laboratory conditions. *Ecology.* **54,927-929.**

Hunt S. & Breuer S. W. (1971)
Isolation of a new naturally occurring halogenated amino acid: monochloro,monobromotyrosine. *Biochim. et Biophys.Acta.* **252,397-400.**

Hunt S. (1984)
Halogenated tyrosine derivatives in invertebrate scleroproteins: Isolation and identification. In: *Methods in Enzymology* (Wold F. and Moldave K. eds.) Vol 107, Posttranslational modifications, Part B, Academic Press.

Hunte W. (1980)
The laboratory rearing of the larvae of the shrimp *Macrobrachium faustinium. Caribbean J. Sci.* **16,(1-4),57-62.**

Jalihal D. R. & Sankolli K. N. (1975)
On the abbreviated metamorphosis of the freshwater prawn *Macrobrachium hendersonayanum* in the laboratory. *J. Karnatak Univ.* **20,283-291.**

Jeuniaux C. (1971)
Haemolymph. In: *Chemical Zoology* (Florkin M. and Scheer B.T. eds.) Ch. 2,Vol VI, part B, Arthropoda, Academic Press. 64-118

John M. C. (1957)
Bionomics and life history of *Macrobrachium rosenbergii* (de Man). *Bull. Cent. Res. Inst. University of Kerala (India).* **Ser. C.,15,93-102.**

Johns D. M., Peters M. E. & Beck A. D. (1980)
International study on *Artemia.* VI. Nutritional value of geographical and temporal strains of *Artemia*: Effects on survival and growth of two species of Brachyuran larvae. In: *The Brine Shrimp Artemia.* (Persoone L. G., Sorgeloos P., Roels O. and Jaspers E. eds.) **Vol. 1,** 291-304. Wettesen, Belgium, Universal Press.

Johns D. M. , Berry W. J. & McLean S. (1981)
Intrnational study on *Artemia* . XXI. Investigations into why some strains of *Artemia* are better food sources than others. Further nutritional work with larvae of *Rithropanopeus harrisii. J. Wld. Maric. Soc.* **12**,303-314.

Johns D. M. (1982)
Physiological studies on *Cancer irroratus* larvae. III. Effects of temperature and salinity on the partioning of energy resources during development. *Mar. Ecol. Prog. Ser.* **8**,75-85.

Johnson D. S. (1967)
Some factors influencing the distribution of freshwater prawns in Malaya. *Proc. Symp. Crustacea Mar. Biol. Assoc. India* **1**,418-433.

Jolles Z. E. (1966)
Biological aspects of bromine and some bromine compounds. In: *Bromine and its compounds* (Jolles Z. E. ed.) Part V, 487-517, Ernest Benn ltd.,London.

Joseph J. D. (1977)
Assessment of the nutritional role of algae in the culture of larval prawns *Macrobrachium rosenbergii. Proc. Wld. Maric. Soc.* **8**, 853-864.

Kamemoto F. I., Kato K.N. & Tucker L.E. (1966)
Neurosecretion and salt and water balance in the Annelida and Crustacea. *Am. Zool.* **6**,213-219.

Kamemoto F. I. & Tullis R.E. (1972)
Hydromineral regulation in decapod crustacea. *Gen. Comp. Endocrinol.* **3**,299-307.

Katz B. (1936)
Neuromuscular transmission in crabs. *J. Physiol.* **87**, 199-220.

Kerkut G.A., Moritz P.M. & Munday K.A. (1961)
Variations of copper concentrations in *Carcinus maenas. Cah. Biol. Marine.* **2**,399-408.

Kerley D. E. & Pritchard A. W. (1967)
Osmotic regulation in the crayfish *Pacifastacus leniusculus,* stepwise acclimated to dilutions of seawater. *Comp. Biochem. Physiol.* **20**,103-113.

Kester D. R., Duedall I. W., Connors D. N. & Pytkowicz R. m. (1967)
Preparation of artificial seawater. *Limnol. Oceanogr.* **112**,176-179.

Kirschner L. B. (1975)
Control mechanisms in crustaceans and fishes. In: *Mechanisms of Osmoregulation in animals.* (Gilles R. ed.) J. Wiley and Sons, New York.

Knowlton R. E. (1974)
Larval development processes and controlling factors in decapod crustacea with emphasis on Caridea. *Thalassia Jugoslavica.* **10**,(1-2), 138-158.

Kon T. (1979)
Ecological studies on larvae of the crabs belonging to the genus *Chionectes.* 1. The influence of starvation on the survival and growth of the Zuwai crab. *Bull. Jap. Soc. Sci. Fish.* **45**, 7-9.

Kullama L. K. (1981)
Moult related changes in transmural sodium transport in freshwater prawn intestine. *Pac. Sci.* **35**,(3),271-272.

Larimer J. L. & Riggs A. F. (1964)
Properties of haemocyanin - 1. The effect of calcium ions on the oxygen equilibrium of crayfish haemocyanin. *Comp. Biochem. Physiol.* **13**,35-46.

Lee C. L. & Fielder D. R. (1984)
Swimming responses to water current stimulus in the freshwater prawn *Macrobrachium australiense* (Holthuis 1950). *Crustaceana.* **46**,(3),249-256.

Leinen R. (1982)
Sodium regulation in a freshwater prawn 2. Sodium fluxes. *Comp. Biochem. Physiol.* **73a**,(2),321-326

Licop S. R. (1983)
Sodium EDTA effects on survival and metamorphosis of *Penaeus monodon* larvae. *Aquaculture* **74**,(3-4),239-248.

Ling S. W. (1969a)
The general biology and development of *Macrobrachium rosenbergii* (de Man). *F.A.O. Fish. Rep.* **57**,(3),589-606.

Ling S. W. (1969b)
Methods of rearing and culturing *Macrobrachium rosenbergii* (de Man). *F.A.O. Fish. Rep.* **57**,(3),607-619.

Ling S. W. & Merican A. B. O. (1961)
Notes on the life and habits of the adults and larval stages of *Macrobrachium rosenbergii* (de Man).*Proc. Indo-Pacif. Fish. Counc.,* 9th session. No.2,55-60.

Lockwood A. P. M. (1961)
'Ringer' solutions and some notes on the physiological basis of their ionic composition. *Comp. Biochem. Physiol.* **2**, 241-289.

Lockwood A. P. M., Croghan P. C. & Sutcliffe D. W. (1976)
Sodium regulation and adaptations to dilute media in Crustacea as exemplified by the isopod *Mesidotea entomon* and the amphipod *Gammarus duebeni.* In: *Perspectives in Experimental Biology* (Davies S. ed.) I. Zoology, 93-106, Pergamon Press, Oxford.

Low E. M. (1949)
Iodine and bromine in sponges. *J. Mar. Res.* **8**, 97.

Low E. M. (1951)
Halogenated amino acids of the bath sponge. *J. Mar. Res.* **10**,239-245.

Lovatt D. L. & Felder D. L. (1988)
Evaluation of the rotifer *Brachionus plicatilis* as a substitute for *Artemia* in feeding the larvae of *Macrobrachium rosenbergii. Aquaculture* **71**, 331-338.

Lyman J. & Fleming R. H. (1940)
Composition of seawater. *J. Mar. Res.* **3**,134-146.

Mackay W. C. & Prosser C. L. (1970)
Ionic and osmotic regulation in the king crab and two other North Pacific crustaceans. *Comp. Biochem. Physiol.* **34**,273-280.

Maddox M. B. & Manzi J. J. (1976)
The effects of algal supplement on static system culture of *Macrobrachium rosenbergii* (de Man)larvae. *Proc. World Maric. Soc.* **7**,677-698.

Magalhaes C. & Walker I. (1988)
Larval development and ecological distribution of central Amazonian Palaemonid shrimps Decapoda, Caridea. *Crustaceana.* **55**,3, 279-292.

Mangum C. P. (1983)
Oxygen transport in the blood. In: *Internal anatomy and physiological regulation. The Biology of the Crustacea.* (Mantel L. H. ed.), Vol. 5,373-429, Academic Press, New York.

Mangum C. P. & Johansen K. (1975)
The colloid osmotic pressures of invertebrate body fluids. *J. Exp. Biol.* **63**, 661-671.

Mangum C. P., Silverthorn S. U., Harris J. L., Towle D. W. & Krill A. R. (1976)
The relationship between pH, ammonia excretion and adaptation to low salinity in the blue crab *Callinectes sapidus. J. Exp. Zool.* **195**,129-136.

Mangum C. P. & Towle D. W. (1977)
Physiological adaptation to unstable environments. *Am. Sci.* **65**,65-75

Mangum C. P. (1986)
Osmoregulation in marine and estuarine animals: Its influence on respiratory gas exchange and transport. *Boll. Zool.* **53**, 1-7.

Mantel L. H. & Farmer L. L. (1983)
Osmotic and Ionic regulation. In: *The Biology of Crustacea* (Mantel L. H. ed.) ch. 2,Vol 5,53-161, Academic press, New York.

Manton S. M. & Anderson D. T. (1979)
Polyphyly and the evolution of the Arthropods. In: *The Origin of the Major Invertebrate Groups.*(House M. R. ed.) Vol. 12 The Systematics Association, Academic Press, New York. 253-268.

Manzi J. J., Maddox M. b. & Sandifer P. A. (1977)
Algal supplement enhancement of *Macrobrachium rosenbergii* (deMan) larviculture.

Martin J. H., Elliott P. D., Anderlini V. C., Girvin D., Jacobs S. A.,
Risebrough R. W., DeLong R. L. & Gilmartin W. G. (1976)
Mercury-Selenium-Bromine imbalance in premature parturient Californian sea-lions. *Mar. Biol.* **35**,(1),91-104.

Martinez-Palacios C. A., Olivera-Novoa M. A. & Chavez- Martinez C. (1985)
A simple apparatus for self-separation of post-larval prawns, *Macrobrachium spp.* in mixed larval rearing. *Aquacult. Fish. Manag.* **16**,(4), 341-348.

Mashiko K. (1984)
Crossing in captivity between the estuarine and upper freshwater individuals of different reproductive traitsin the long armed prawn *Macrobrachium nipponense. Jap. J. Ecol.* **34**,(2) 229-232.

Mashiko K. (1990)
Diversified egg and clutch sizes among local populations of *Macrobrachium nipponense* (de Haan). *J. Crust. Biol.* **10**, (2), 306-314.

Mauro N. A. & Malecha S. R. (1984a)
The effects of hypoxia on blood pH and lactate levels in *Macrobrachium rosenbergii* (de Man). *Comp. Biochem. Physiol.* **77a**,(4),627-630.

Mauro N. A. & Malecha S. R. (1984b)
Intraspecific comparison of haemocyanin and the effects of hypoxia on oxygen consumption in *Macrobrachium rosenbergii* (de Man). *Comp. Biochem. Physiol.* **77a**,(4),631-633.

Maw G. A. & Kempton R. J. (1982)
Bromine in soils and peats. *Plant and Soil.* **65**, (1), 103-109.

McCaffrey M. A., Lazar B. & Holland H. D. (1987)
The evaporation path of seawater and the coprecipitation of Br^- and K^+ with halite. *J. Sedimentol. Petrol.* **57**,(5),928-937.

McConaugha J. R. (1985)
Nutrition and larval growth. In *Crustacean Issues 2. Larval Growth.* (Schram F. R. ed.). A.A. Balkema. Rotterdam.127-154.

McMahon B. R. & Wilkens J. L. (1983)
Ventilation, Perfusion, and Oxygen uptake. In: *The Biology of Crustacea* (Mantel L. H. ed.) ch. 6,Vol 5, 53-161, Academic press, New York.

McNamara J. C. (1987)
The time course of osmotic regulation in the freshwater shrimp *Macrobrachium olfersii* (Wiegmann) Decapoda, Palaemonidae. *J. Exp. Mar. Biol. Ecol.* **107**,245-251.

McNamara J. C., Salomao L. C. & Riberio E. A. (1990)
The effect of eyestalk ablation on haemolymph osmotic and ionic concentrations during acute salinity exposure in the freshwater shrimp *Macrobrachium olfersii. Hydrobiologia.* **199b**,(3),193-200.

Minamizawa A. & Morizane T. (1970)
Report on study about cultivation techniques for freshwater shrimp. *Eheim Prefecture Fish. Lab. Japan* (Translated from the Japanese, Bur. Comm. Fish., U.S. Dept. Interior).

Moran W. M. & Pierce S. K. (1984)
The mechanism of crustacean salinity tolerance: Cell volume regulation by K^+ and glycine effluxes. *Mar. Biol.* **81**, (1), 41-46.

Morcos S. A. (1973)
A table for the ionic composition of seawater based on 1967 atomic weights. *J. Conseil. Int. Expl. Mer.* **35**,94-95.

Moreira G. S., Ngan P. V., Moreira P. S. & Shumway S. E. (1988)
The effect of salinity on the osmo-ionic regulation of *Macrobrachium carcinus* (L.). *Comp. Biochem. Physiol.* **91a**,(1),105-108.

Moreira G. S., McMamara J. C., Shumway S. E. & Moreira P. S. (1983)
Osmoregulation and respiratory metabolism in Brazilian *Macrobrachium. Comp. Biochem. Physiol.* **74a**, (1), 57-62.

Morel F. M. M., Rueter J. G., Anderson D. M. & Guillard R. R. L. (1979)
AQUIL - A chemically defined phytoplankton culture medium for trace metal studies. *J. Phycol.* **15**,135-141.

Morris S., Tyler-Jones R. & Taylor E. W. (1986)
The regulation of haemocyanin oxygen affinity during emergence of the crayfish *Austropotamobius pallipes.* 1. An *in vitro* investigation of the interactive effects of calcium and L-(+)-Lactate on oxygen affinity. *J. Exp. Biol.* **121**,315-326.

Morris S., Taylor A. C. & Bridges C. R. (1988)
Response of haemolymph oxygen affinity to simultaneous salinity and oxygen stress in the intertidal prawn, *Palaemon elegans* (Rathke). *Comp. Biochem. Physiol.* **90a**,(1),31-39.

Morris S. (1990)
Organic ions as modulators of respiratory pigment function during stress. *Physiol. Zool.* **63**,(2), 253-287.

Nair S. R. S., Goswami U. & Goswami S. C. (1977)
The effect of salinity on the survival and growth of the laboratory reared larvae of *Macrobrachium rosenbergii. Mahasagar.* **10**,(3-4),139-144.

Nelson S. D., Armstrong D. A., Knight A. W. & Li H. W. (1977)
The effects of temperature and salinity on the metabolic rate of juvenile *Macrobrachium rosenbergii* (Crustacea: Palaemonidae). *Comp. Biochem. Physiol.* **56a**, 533-537.

Neville A. C. (1975)
Biology of the Arthropod cuticle. Springer-Verlag, Berlin.

New M. B. (1988)
Freshwater prawns: Status of global aquaculture, 1987. NACA Technical manual No.6. A World Food Day publication of the Network of Aquaculture Centres in Asia. Bangkok, Thailand. 58pp.

New M. B. (1990)
Freshwater prawn culture - a review. *Aquaculture* **88**,99-143.

New M. B. & Singholka S. (1985)
Freshwater prawn farming. A manual for the culture of *Macrobrachium rosenbergii. F.A.O. Fish. Tech. Paper.* **225**, revision 1. 118 pp.

Olney C. E., Schauer P. S. , McLean S., Li Y. & Simpson K. L. (1980)
Comparison of chlorinated hydrocarbons and heavy metals in five different strains of newly hatched *Artemia* and laboratory reared marine fish. In: *The Brine Shrimp Artemia.* (Persoone L. G., Sorgeloos P., Roels O. and Jaspers E. eds.) **Vol. 3**, 343-352. Wettesen, Belgium, Universal Press.

Ortman A. E. (1902)
The geographical distribution of freshwater decapods and its bearing upon ancient geography. *Proc. Am. Phil. Soc.* **41**,267-400.

Pannikar N. K. (1941)
Osmoregulation in some palaemonid prawns. *J. Mar. Biol. Assoc. U.K.* **25**,317-359.

Parry G. (1954)
Ionic regulation in the palaemonid prawn *Palaemon (=Leander) serratus. J. Exp. Biol.* **31**,(4),601-613.

Parry G. (1957)
Osmoregulation in some freshwater prawns. *J. Exp. Biol.* **34**,417-423.

Parry G. & Potts W. T. W. (1965)
Sodium balance in the freshwater prawn, *Palaemonetes antennarius. J. Exp. Biol.* **42**,415-421.

Passano L. M. (1960)
Moulting and its control. In: *The Physiology of Crustacea* (Waterman T. H. ed.) Vol 1, 413-536, Academic Press, New York.

Paul A. J., Paul J. M., Shoemaker P. A. & Feder H. M. (1979)
Prey concentrations and feeding response in laboratory reared stage one zoea of King crab, Snow crab and Pink shrimp. *Trans. Am. Fish. Soc.* **108**,440-443.

Peebles J. B. (1977)
A rapid technique for moult staging in live *Macrobrachium rosenbergii. Aquaculture.* **12**, 173-180.

Perdue J. A. & Nakamura R. (1976)
The effect of salinity on the growth of *Macrobrachium rosenbergii. Proc. World Maric. Soc.* **7**,647-654.

Pillai S. M., Verghese P. V., Ravichandran P. & Roy A. K. (1987)
Effect of thyroxine on growth and development in *Penaeus monodon* (Fabr.). *Indian J. Anim. Sci.* **57**,(3),241-245.

Prakash S. (1988)
On the comparative efficiency of two cuture media for the larval rearing of giant freshwater prawn, *Macrobrachium rosenbergii. J. Zool. Res.* **1**,(1),33-37.

Prakash S., Wishard S. K. & Saxena R. K. (1987)
On breeding and rearing the freshwater prawn *Macrobrachium choprai* in closed water systems. *Indian J. Fish* **34**, (4), 374-381.

Provenzano A. J. & Goy J. W. (1976)
Evaluation of a sulphate lake strain of *Artemia* as a food source for larvae of grass shrimp, *Palaemonetes pugio. Aquaculture.* **9**,343-350.

Rabanal H. R. & Soesanto V. (1985)
The world fishery and culture of *Macrobrachium* and related species.ASEAN/UNDP/FAO regional small scale fisheries development project. *ASEAN/SF/85/ Tech 1.* 16pp.

Read G. H. L. (1984)
Intraspecific variation in the osmoregulatory capacity of larval and postlarval juveniles and adult *Macrobrachium petersii* (Hilgendorff). *Comp. Biochem. Physiol.* **78a**, (3), 501-506.

Redmond J. R. (1971)
Blood respiratory pigments - Arthropoda. In: *Chemical Zoology* .Vol. VI,119-144, (Florkin M. and Scheer B. T. eds.), Academic Press.

Reeve M. R. (1969)
Growth and metamorphosis and energy conversion in the larvae of the prawn *Palaemon serratus. J. Mar. Biol. Assoc. U.K.* **49**,77-96.

Rheinhold J. G. (1975)
Trace elements - a selective survey. *Clin. Chem.* **21**, (4), 476-500.

Richards A. G. (1951)
The integument of Arthropods. University of Minnesota Press.

Roberts M. H. Jr. (1974)
Larval development of *Pagurus longicarpus* (Say) reared in the laboratory. V. Effect of diet on survival and moulting. *Biol. Bull.* **146**, 67-77.

Robertson J. D. (1937)
Some features of the calcium metabolism of the shore crab.(*Carcinus maenas.* Pennant).*Proc. Roy. Soc. London. Ser. B,* **124**, 162-182.

Robertson J. D. (1953)
Further studies on ionic regulation in marine invertebrates. *J. Exp. Biol.* **30**,277-296.

Robertson J. D. (1960)
Osmotic and Ionic regulation. In: *Physiology of the Crustacea.* (Waterman T. H. ed.) Vol. 1, Academic Press.

Roche J., Fontaine M. & Leloup J. (1963)
Halides. in *Comparitive Biochemistry.* Vol 5c, (Florkin M. and Mason H.S. eds.), Academic Press, New York. 493-547.

Sandifer P. A., Hopkins J. S. & Smith T. I. J. (1974)
Development of a crustacean mariculture programme at South Carolina's Marine Resources Research Institute. *Proc. World. Maric. Soc.* **5**,431-439.

Sandifer P. A., Hopkins J. S. & Smith T. I. J. (1975)
Observations on salinity tolerance and osmoregulation in laboratory reared *Macrobrachium rosenbergii* postlarvae. *Aquaculture* **6**,103-114.

Sandifer P. A. & Smith T. I. J. (1985)
Freshwater Prawns. In: *Crustacean and Mollusc aquaculture in the United States.* (Huner J. V. and Brown E. E. eds.) AVI, Westport, Conneticut.USA. 125pp.

Sargent J., Henderson R. J. & Tocher D. R. (1989)
Chapter 4: The Lipids. In *Fish Nutrition,* 2nd Edition, Academic Press,153-217.

Schoffeniels E. (1976)
Adaptations with respect to salinity. *Biochem. Soc. Symp.* **41**,179-204.

Schoffeniels E. & Gilles R. (1970)
Osmoregulation in aquatic arthropods. In: *Chemical Zoology* (Florkin M. and Scheer B. T.) Vol.V,255-286, Academic Press.

Sharma M. L. (1969)
Trigger mechanisms of increased urea production by the crayfish, *Orconectes rusticus,* under osmotic stress. *Comp. Biochem. Physiol.* **30**,309-321.

Shokita S. (1973)
Abbreviated development of the freshwater prawn , *Macrobrachium shokitai* (Fujino & Baba) from Iriomote Island of the Ryukyus. *Annotnes. Zool. Jap.* **46**, 111-126.

Shokita S. (1977)
Abbreviated metamorphosis of land locked freshwater prawn *Macrobrachium asperulum* from Taiwan. *Annotnes Zool. Jap.* **50**,110-122.

Sick H. & Gersonde K. (1969)
Method for continuous registration of O_2-binding curves of hemoproteins by means of a diffusion chamber. *Analyt. Biochem.* **32**,362-376

Singh T. (1977)
Osmotic and ionic regulation in *Macrobrachium rosenbergii* (deMan), 1879 (Decapoda: Caridea). MSc. Thesis, University of Malaya,56pp.

Singh T. (1980)
The isosmotic concept in relation to the aquaculture of the giant prawn *Macrobrachium rosenbergii. Aquaculture* **20**, 251-256.

Smith D. M. & Dall W. (1982)
Blood protein, blod volume and extracellular space relationships in two *Peneaus spp.* (Decapoda: Crustacea). *J. Exp. Mar. Biol. Ecol.* **63**, 1-15.

Smith T. I. J., Sandifer P. A. & Jenkins W. E. (1982)
Growth and survival of prawns *Macrobrachium rosenbergii,* pond reared at different salinities. *Dev. Aquat. Fish. Sci.* **10**,191-202.

Soevik T. & Braekkan O.R. (1979)
Fluoride in Antarctic Krill (*Euphausia superba*) and Atlantic Krill (*Meganyctiphanes norvegica*). *J. Fish. Res. Board Can.* **36**,1414-1416.

Sollaud E. (1923)
Le developpment larvaire des *Palaemonidae.* I. Partie descriptive. La condensation progressive de l'ontogenese. *Bull. Biol. Fr. Belg.* **57**,509-603.

Spaargaren D. H. (1983)
Osmotically induced changes in copper and iron concentrations in three euryhaline crustacean species. *Neth. J. Sea. Res.* **17**,(1), 96-105.

Spaargaren D. H. (1988)
Low temperature halogen accumulation in the brown shrimp, *Crangon crangon. Comp. Biochem. Physiol.* **90a**, (3), 397-404.

Spearman R. I. C. (1973)
The integument, a textbook of skin biology. Cambridge University Press.

Spotte S. (1979)
Fish and Invertebrate Culture. John Wiley and Sons, New York. 179pp.

Spotts D. G. (1983)
Oxygen consumption and whole body lactate accumulation during progressive hypoxia in the tropical freshwater prawn, *Macrobrachium rosenbergii* (de Man). *J. Exp. Zool.* **226**,19-27.

Stephenson M. J. & Knight A. W. (1980)
The effect of temperature and salinity on oxygen consumption of post-larvae of *Macrobrachium rosenbergii* (de Man) (Crustacea : Palaemonidae). *Comp. Biochem. Physiol.* **67a**, 699-703.

Stern S., Borut A. & Cohen D. (1987)
Osmotic and ionic regulation of the prawn *Macrobrachium rosenbergii* (de Man) adapted to varying salinities and ionic concentrations. *Comp. Biochem. Physiol.* **86a**, 2, 373-379.

Stevenson J. R. (1961)
Polyphenol oxidase in the tegumental glands in relation to the moulting cycle of the isopod crustacean *Armadillidium vulgare. Biol. Bull.* **121**,554-560.

Stevenson J. R. (1985)
Dynamics of the integument. In:*The Biology of Crustacea.* (Bliss D. E. and Mantel L. H. eds.). Ch.1, Vol 9, Academic Press,1-42.

Stevenson J. R. & Schneider R. P. (1962)
Tyrosinase activity of organs containing tegumental glands in the crayfish. *J. Exp. Zool.* **150**,17-25

Strickland J. D. H. & Parsons T. R. (1972)
A practical handbook of seawater analysis. 2nd edition. *Bull. 167, Fish Res. Board Can.* 310pp.

Subramian P. & Krishnamurhty K. (1986)
Effect of salinity and body size on metabolism and growth of juvenile Penaeid prawns. *Indian J. Exp. Biol.* **24**,773-778.

Szewielow A. (1981)
Fluoride in krill (*Euphausia superba* Dana). *Meeresforsch.* **28**, (4), 224-246.

Szyper J. P. (1989)
Nutritional depletion of the aquaculture feed organisms *Euterpina acutifrons* , *Artemia spp.* and *Brachionus plicatilis* during starvation. *J. World Aquacult. Soc.* **20**, 3, 162-169.

Tait R. V. (1968)
Elements of Marine Ecology. Butterworths, London.

Tan C. H. & Choong K. Y. (1981)
Effect of hyper-osmotic stress on haemolymph protein, muscle ninhydrin positive substances and free amino acids in *Macrobrachium rosenbergii. Comp. Biochem. Physiol.* **70**,(4), 485-490.

Tansakul R. (1983)
Progress in Thailand rearing larvae of the giant prawn *Macrobrachium rosenbergii* in salted water. *Aquaculture* **31**,95-98.

Taylor A. C. & Spicer (1988)
Functional significance of a partial-emersion response in the intertidal prawn *Palaemon elegans* (Crustacea: Palaemonidae) during environmental hypoxia. *Mar. Ecol.-Prog. Ser.* **44**,141-147.

Taylor E. W., Butler P. J. & Al-Wassia A. (1977)
Some responses of the shore crab, *Carcinus maenas* (L.) to progressive hypoxia at different acclimation temperatures and salinities. *J. Comp. Physiol.* **122**,391-402.

Tentori E. & Lockwood A. P. M. (1990)
Haemolymph magnesium levels in some oceanic Crustacea. *Comp. Biochem. Physiol.* **95a**, (4), 545-548.

Thompson T. G. & Robinson R. J. (1932)
Chemistry of the sea. In: *Properties of Seawater. Bull. Nat. Res. Counc. Wash.* **85**,95.

Treherne J. E. (1966)
The Neurochemistry of Arthropods. Cambridge University Press.

Truchot J-P. (1973)
Fixation et transport de l'oxygene par le sang de *Carcinus maenas:* variations en rapport avec diverses conditions de temperature et de salinite. *Neth. J. Sea Res.* **7**,482-495.

Truchot J-P. (1975)
Factors controlling the *in vitro* and *in vivo* oxygen affinity of the haemocyanin in the crab *Carcinus maenas* (L.). *Resp. Physiol.* **24**,173-189.

Tunsutapanich A. (1980)
The use of rock salt, sea salt, rock salt brine and sea salt stock solution for larval culture of *Macrobrachium rosenbergii. UNDP/FAO for the expansion of freshwater prawn farming working paper.* THA/75/008/80/WP/18, Rome, FAO.

Tunsutapanich A. (1981)
Macrobrachium breeding in mixed salt waters. *Thai Fish. Gaz.* **34**, (1), 43-55.

Underwood E. J. (1977)
Trace Elements in Human and Animal Nutrition. Academic Press, London.

Uno Y. & Soo K. C. (1969)
Larval development of *Macrobrachium rosenbergii* (de Man) reared in the laboratory. *J. of the Tokyo University of Fisheries* **55**, (2), 179-180.

Uno Y. & Yagi H. (1980)
Combination effects of the temperature and salinity factors on the larval growth of *Macrobrachium rosenbergii* (de Man). *UMI/MER* **18**, (4), 171-178.

Venchicov A. I. (1974)
Zones of display of biological and pharmacotoxicological action of trace elements. In: *Trace Element Metabolism in Animals-2.* (Hoekstra W. G., Suttie J. W., Gauther H. E. and Mertz W. eds.) University Park Press, Baltimore. 295-310.

Vigh D. A. & Dendinger J. E. (1982)
Temporal relationships of postmoult deposition of calcium, magnesium, chitin and protein in the cuticle of the Atlantic blue crab *Callinectes sapidus* Rathbun. *Comp. Biochem. Physiol.* **72a**, (2), 365-369.

Vinogradov A. P. (1953)
The Chemical Composition of Marine Organisms. Vol II, Yale University Sears Foundation Marine Research, New Haven, Connecticut.

Visser J. H. & Minks A. K. (1982)
The insect on the plant - a closer look. *Proc. 5th Int. Symp. on Insect - Plant Relationships.* Wageningen, Netherlands (1 -4 March 1982), p.3-18.

Vovelle J., Grasset M. & Truchet M. (1983)
Halogenated compounds and mineral elements acting in the consolidation of the setae of *Petta pusilla. Cah. Biol. Mar.* **24**, (3), 309-325.

Vovelle J., Grasset M. & Truchet M. (1989)
Biomineralisation of the jaws of *Hyalinoecia tubicola* (Mueller). *Ann. Inst. Oceanogr. Paris. (Nouv. Ser.)* **65**, 15-36.

Walters N. J. & Uglow R. F. (1981)
Haemolymph magnesium and relative heart activity of some species of marine decapod crustaceans. *J. Exp. Mar. Biol. Ecol.* **55**,255-265.

Watanabe T., Ohta M., Kitajima C. & Fujita S. (1982)
Improvement of dietary value of brine shrimp *Artemia salina* for fish larvae by feeding them on w3 Highly Unsaturated Fatty Acids. *Bull. Jap. Soc. Sci. Fish.* **48**,(12),1775-1782.

Webb J. S. (1973)
Bromo compounds from marine organisms. In: *Food and Drugs from the Sea.* (Worthen L.R. ed.) Marine Technology Society, Washington.

Wehrtmann I. S. & Dittel A. I. (1990)
Utilisation of floating mangrove leaves as a transport mechanism of estuarine organisms, with emphasis on decapod Crustacea. *Mar. Ecol. Prog. Ser.* **60**, 67-73.

Weiland A. L. & Mangum C. P. (1975)
The influence of environmental salinity on haemocyanin function in the blue crab*Callinectes sapidus. J. Exp. Zool.* **193**, 265-274.

Wheatley M. G. & McMahon B. R. (1982a)
Responses to hypersaline exposure in the euryhaline crayfish *Pacifastacus leniusculus.* I. The interaction between ionic and acid-base regulation. *J. Exp. Biol.* **99**, 425-446.

Wheatley M. G. & McMahon B. R. (1982b)
Responses to hypersaline exposure in the euryhaline crayfish *Pacifastacus leniusculus*. II. Modulation of haemocyanin oxygen binding *in vitro* and *in vivo*. *J. Exp. Biol.* **99**, 447-467.

Wickins J. F. (1972)
The food value of brine shrimp *Artemia salina* (L.) to the larvae of the prawn, *Palaemon serratus*. (Pennant). *J. Exp. Mar. Biol. Ecol.* **10**, 151-170.

Wickins J. F. (1976)
The tolerance of warm-water prawns to recirculated water. *Aquaculture* **9**, 19-37.

Wickins J. F. (1984)
The effect of reduced pH on carapace calcium, strontium and magnesium levels in rapidly growing prawns *Penaeus monodon*. *Aquaculture* **41**, 49-60.

Wickins J. F. & Beard T. W. (1974)
Observations on the breeding and growth of the giant freshwater prawn *Macrobrachium rosenbergii* (deMan) in the laboratory. *Aquaculture* **3**,159-174.

Willis S. A. & Berrigan M. E. (1976)
Growout of the giant Malaysian prawn, *Macrobrachium rosenbergii*, in earthen ponds in central florida. *Fla. Dept. Natl. Res.* 38pp.

Winkler A. (1986)
Effect of inorganic seawater constituents on branchial Na-K-ATPase activity in the shore crab *Carcinus maenas*. *Mar. Biol.* **92**, (4), 537-544.

Winkler L. R. (1969)
Distribution of organic bromine compounds in *Aplysia californica* (Cooper1863). *The Veliger* **11**, 268-271.

Wolf A. V., Brown M. G. & Prentiss P. G. (1980)
Concentrative properties of aqueous solutions: Conversion tables. In: *CRC Handbook of Chemistry and Physics, 81st edition.* CRC Press, West Palm Beach, Florida. p D-210.

Wong T. M. & Freeman R. F. H. (1967)
Haemolymph concentrations of two species of New zealand freshwater crayfish in relation to the concentration of their external media. *Comp. Biochem. Physiol.* **55a**,13-16.

Wright D. A. (1980)
Calcium balance in premoult and postmoult *Gammarus pulex* (Amphipoda). *Freshwater Biology* **10**,571-579.

Wright D. A., Zanders I. P. & Pait A. (1984)
Ionic regulation in three species of *Uca*: A comparative study. *Comp. Biochem. Physiol.* **78a**,(1),175-179.

Wulff R. E. (1982)
The experience of a freshwater prawn farm in Honduras, Central America. In: *Giant Prawn Farming,* (New M. B. ed.) Elsevier, Amsterdam, 445-448.

Yambot A. V. & Vera Cruz (1986)
Larval rearing of *Macrobrachium rosenbergii* (de Man) in brine solution and sea salt. In: *Proc. First Asian Fisheries Forum* (Maclean J. L. et al eds.) Asian Fisheries Soc., Manila, Philippines.

Yonge C. M. (1932)
On the nature and permeability of chitin. I. The chitin lining the foregut of decapod Crustacea and the function of the tegumental glands. *Proc. Roy. Soc.* **B111,** 298-329.

Zanders I. P. (1980)
The control of magnesium and sulphate excretion in *Carcinus maenas.* (L.). *Comp. Biochem. Physiol.* **66a,** 68-76.

Zanders I. P. (1981)
Control and dynamics of ionic balance in *Carcinus maenas.* (L.). *Comp. Biochem. Physiol.* **70a,** 457-468.

Zuckerkandl E. (1960)
Hemocyanine et cuivre chez un crustace decapode, dans leurs rapports avec le cycle d'intermue. *Annls. Inst. Oceanogr., Monaco* **38,**1-122.

Appendix 1

Adult *Macrobrachium rosenbergii* maintenance diet.

1.1 Diet

Ingredient	% inclusion (dry weight of diet)
herring meal	17
dried bakers yeast	3
wheat meal	15
soya meal	20
rice bran	26
gelatin	4
agar	7
cod liver oil	2.5
vitamin premix	2
mineral premix	2
'Finn stimm'	1.5

1.2 Vitamin premix

Vitamin	inclusion (g/100g of premix)
Thiamine-HCl	0.1500
Riboflavin	0.5000
Pyroxidine-HCl	0.1500
Niacinamide	2.0000
Pantothenic acid	0.7500
Inositol	10.0000
Biotin	0.0150
Folic acid	0.0375
P-aminobenzoic acid	1.0000
Ascorbic acid	25.0000
Menadione (K3)	0.1000
ß-carotene	0.1000
Cholecalciferol	0.0150
Cyancobalamine(B12)	0.0010
Choline chloride	20.0000
a-tocopherol acetete*	1.0000
a-cellulose powder	39.1815

* added to oil in diet

1.3 mineral premix

mineral		% inclusion (g/100 g premix)
$CaHPO_4$	$2H_2O$	72.776
$MgSO_4$	$7H_2O$	12.75
NaCl		6.00
KCl		5.00
$FeSO_4$	$7H_2O$	2.50
$ZnSO_4$	$7H_2O$	0.55
$CuSO_4$	$8H_2O$	0.0785
$MnSO_4$	$4H_2O$	0.2538
$CoSO_4$	$7H_2O$	0.0478
$CaIO_3$	$6H_2O$	0.0295
$CrCl_3$	$6H_2O$	0.0128

1.3 proximate analysis

	% by wet weight
protein	37.8
carbohydrate*	33.5
lipid	4.6
crude fibre	4.4
moisture	9.7
ash	10.0

* carbohydrate = 100 - (other constituents)

Appendix II

Homemade artemia enrichment compounds

1) Watanabe *et al.* (1982) 'mayonnaise'.

0.3g	**egg yolk**	1.376
1.5g	**fish oil**	6.881
20.0g	**seawater**	91.743

To the fish oil is added vitamin E, at 1.5 % by weight of oil.

The oil and egg yolk are emulsified in a liquidiser, and the seawater gradually added in. The resulting emulsion is added at $2cm^3.dm^{-3}$. This gives an oil concentration of $0.05g.dm^{-3}$. Enrichment is for 8 hours at an artemia concentration of approximately $300,000.dm^{-3}$.

2) Homemade enrichment formulae

	Formula 1	Formula 2
Fish oil	60 %	33%
Tween 80	10 %	10%
Water	28 %	55%
Soya lecithin	2 %	2%

To the oil is added:

vitamin E	1.5 % by weight of oil
cholesterol	2.0 %
astaxanthin	2-3 ml

All ingredients except the water are mixed in a liquidiser. The water is slowly added until the desired consistency is obtained. The enrichment should be fluid enough to pour. Addition of the enrichment solution should be calculated at the rate of 0.1 g $oil.dm^{-3}$. The concentration of this enrichment compound is 0.3 $g.dm^{-3}$ of artemia rearing water, this gives an oil concentration of 0.1 $g.dm^{-3}$. This concentration is double that of the formula of Watanabe *et al.* (1982)

Appendix III

Postlarval separator

The separator was tested four times with mixed batches of postlarvae and larvae. Each trial was run for approximately five minutes, before the separator was switched off and the larvae and postlarvae were counted.

1.0 Results

	larvae	postlarvae	% separation
TOTAL	1572	472	
larvae trap	1524	40	97.4
postlarvae trap	48	432	90.0
% separation	96.9	91.5	
TOTAL	544	243	
larval trap	507	86	83.0
postlarval trap	37	157	76.4
% separation	93.2	64.6	
TOTAL	1597	250	
larval trap	1465	37	97.5
postlarval trap	132	213	61.7
% separation	91.7	85.2	
TOTAL	2073	313	
larval trap	1999	64	96.8
postlarval trap	74	249	70.3
% separation	96.4	79.6	

The postlarval separator appears to give good separation between the larvae and postlarvae. The figure of 82% quoted for the separator of **Martinez-Palacios *et al.* (1985)** represented the percentage of postlarvae that were removed from the culture tank over a 24 hour period. Only postlarvae were used in their experiment, although this would not have affected the results, as the design would only have attracted postlarvae. The ratio of postlarvae to larvae will also have an effect on the efficiency of the separator. This was not tested in these trials with the larvae present at approximately three times the

number of postlarvae. This was considered to be a worst case situation, and postlarval separation would be improved if their numbers were greater. It seems that because the separation method is an active process efficiency is linked to a large mass of animals in a relatively small volume. Again this might be a constraint if the device were to be scaled up to commercial size.

The speed of separation of the postlarvae and larvae is the attraction of the separator used in this work. With a separation time of approximately 5 minutes, the larvae and postlarvae can be processed very rapidly and therefore minimize stress. The applicability of this design to a commercial situation is hard to assess. In order for correct functioning the larvae and postlarvae are required to be present in a relatively small volume of water. This would involve the removal of all the larvae and postlarvae from a tank, and may not be desirable or feasible commercially.