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**DEVELOPMENT OF HATCHERY TECHNIQUES FOR THE MUD  
CRAB *SCYLLA SERRATA* (FORSKÅL) IN SOUTH AFRICA**

**ONTWIKKELING VAN BROEDHUISTECHNIEKEN VOOR DE  
MANGROVE KRAB *SCYLLA SERRATA* (FORSKÅL) IN ZUID  
AFRIKA**

by/door

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# Chapter 1

## General introduction

Most aquaculture texts begin by presenting the world fisheries yield (which is fast approaching its sustainable limit) and comparing it to the healthy growth of aquaculture production (FAO, <http://www.fao.org/docrep/>). This often leads to the assumption that aquaculture's role is to provide the world's poor with fish protein in the future. There is indeed much scope for expanding the production of (particularly) omnivorous species. Traditional farming of cyprinid species in China in particular, still constitutes the bulk of world aquaculture production (FAO, Fishstat 2002). However the nature of the industry is changing. Modern aquaculture (like most agro-industry) is profit driven and governed by free-market principles: *i.e.* when the fishery for a species can no longer satisfy market demand, the price rises and this makes farming commercially viable (Jolly and Clonts 1993). The more expensive the species, the more attractive it is to farm and the concept of aquaculture being a producer of cheap protein is giving way to a dynamic industry which targets specific market segments (Wickins and Lee 2002). Even *Tilapia*, once termed the “aquatic chicken”, is now grown to a large size, filleted and served in the world's finest restaurants (Urch 1996). The value of the luxury seafood market and thus the scope for aquaculture is illustrated by the fact that in the USA in 1992, seafood was the second biggest import in value after oil (National Research Council Committee 1992).

The aquaculture industry is constantly diversifying to meet the demand for new species. This demand is mostly driven by the decline of traditional fisheries. In Europe for instance, the collapse of the seabass (*Dicentrarchus labrax*), seabream (*Sparus aurata*) and turbot (*Scophthalmus maximus*) fisheries in the Mediterranean has led to the establishment of successful aquaculture production of these species (Brown 2002; Theodorou 2002). The most dramatic example is cod (*Gadus morhua*) which was once considered the “stock” fish of the Atlantic. In Norway, production from farming may soon outstrip fisheries yield (Gallagher 2003). New species are also sought for other reasons. In the 1980's, for example, the unregulated boom in intensive penaeid shrimp culture contributed to the spreading of disease,

particularly the white spot syndrome virus (WSSV) epidemic, which by 1992 had decimated the industry in Taiwan. The epidemic then spread to intensive shrimp farms in the rest of Asia and the Americas (Hill 2002). This led to a scramble for new candidate species, resistant to the virus which could replace shrimp. The maturing of the aquaculture industry has also affected market dynamics. Atlantic salmon is now produced in such volumes that low prices are forcing farmers to look to “new”, more profitable species.

Not only is the number of species being cultured increasing, but as technology and markets become more accessible, the areas in the world where aquatic species are being farmed is expanding. Aquaculture in South Africa is a relatively recent activity, but after an initially slow teething period it is steadily growing into a dynamic, sustainable new industry (Hecht 1999). There have been a number of expensive mistakes in the past which have highlighted the importance of species selection and market dynamics. The production of low value species such as carp (*Cyprinus carpio*), tilapia (*Oreochromis* and *Tilapia* spp.) and catfish (*Clarias gariepinus*) has not been as profitable as expected (Hecht and Britz 1990), whereas there has been successful development of high value species such as oysters (*Crassostrea* spp.), mussels (*Mytilus galloprovincialis*), trout (*Oncorhynchus mykiss*) and a variety of ornamental fish (Hecht 1999). The most illustrative example is the success of the abalone (*Haliotis midae*) industry which has now grown to 12 farms with an estimated investment of US\$ 12 million (Sales and Britz 2001) and a projected production of 750 tons for 2003 (Peter Britz, Department of Ichthyology and Fisheries Science, Rhodes University, Grahamstown, South Africa, pers. comm.). All farmed abalone are exported. The prospects for high intensity, small scale aquaculture in South Africa are good and there is a constant search for new, high value species which would be suitable for culture in the country.

Crustaceans are amongst the most highly valued of luxury foods, but of the 26,000 species of crustaceans (Ruppert and Barnes 1994) only penaeid shrimps, mitten crab (*Eriocheir sinensis*) and freshwater prawns (*Macrobrachium* spp.) are being produced on an industrial scale (Wickins and Lee 2002). In South Africa approximately 150 tons of *Fenneropenaeus indicus* are produced from two commercial farms and there is some experimental production of introduced *Macrobrachium rosenbergii* and *Cherax tenuimanus* (Hecht 1999). There has been increasing interest in crab farming worldwide due to growing markets. The farming of mitten crabs (*Eriocheir* spp.) is already established in China and there is some interest in swimming crabs (*Portunus* spp.) and the king crab (*Mithrax spinosissimus*) (Wickins and Lee 2002). The mud crabs (*Scylla* spp.) are highly sought after luxury seafood items in East Asia (particularly in Japan, Taiwan, Hong Kong and Singapore) where live crabs (especially gravid females)

command premium prices (Keenan 1999; Agbayani 2001). There is also a growing market in the USA for frozen, soft-shelled mud crab and the demand for mud crab meat for value added products is expanding internationally (Cholik 1999; Tan 1999; Keenan 1999; Wickins and Lee 2002).

*Scylla* spp. are large, euryhaline crabs which as adults inhabit sheltered coastal habitats across the Indo-West Pacific. Larger populations are usually associated with mangrove forests, particularly in estuaries (Le Vay 2001) where they tolerate a wide range of environmental conditions (Hill 1979). They are primarily carnivorous, eating benthic molluscs and crustaceans (Hill 1979), but are also opportunistic scavengers and will eat a wide variety of animal protein and even vegetable matter such as filamentous algae (Williams and Primavera 2001) and cooked maize (Rodríguez *et al.* 2003). The crabs are mostly nocturnal or crepuscular, hiding during the day in burrows and foraging on mud flats from late afternoon to early morning, particularly during flood tides (Dat 1999a; Barnes *et al.* 2002). Maturation and spawning is continuous throughout the year with seasonal peaks in certain areas (Le Vay 2001). Adults mate inshore when the exoskeleton of the female is still soft after moulting. Females undergo up to three post-pubertal moults. Mated, ovigerous females generally migrate offshore where they extrude the eggs, attach them to the pleopods and incubate them till they hatch (Brick 1974; Hill 1978; Perrine 1979). Females then return inshore and can spawn up to three times without needing to mate again. Little is known about the oceanic phases of the larvae (Fielder 1995) except that the larvae develop through five pelagic zoeal stages after which they recruit into sheltered, shallow water habitats as semi-benthic megalopae (Brick 1974; Hill 1975 1979). The megalopae settle amongst algae, sea grasses and mangrove roots where they metamorphose into juveniles. Juveniles migrate from inter-tidal to sub-tidal habitats (Le Vay 2001) over the course of their 15-17 moults to maturity (Robertson 1996). Keenan *et al.* (1998) recently revised the genus and identified four distinct species: *S. serrata*, *S. tranquebarica*, *S. olivacea* and *S. paramamosain*. *S. serrata* is more abundant in high salinity habitats than the other three species and females migrate further offshore, often as far as the continental shelf (Heasman and Fielder 1983; Hill 1994). *S. serrata* is also the largest of the four species, reaching a maximum of 24 cm carapace width (Le Vay 2001) and exhibits the highest growth rate: growth of South African *S. serrata* in the wild can be described by the von Bertalanffy equation of the form  $L_t = 188.2(1 - e^{-1.02(t+0.21)})^{3.06}$  (Robertson 1987). Because of their large size *S. serrata* also fetch the highest market price of the four species in Eastern markets (Agbayani 2001).

Mud crabs are easily caught in traps or nets and remain alive for considerable periods after capture (Gillespie and Burke 1992; Wickins and Lee 2002). Fishing can provide an important source of income to rural communities (Keenan 1999) and small-scale fisheries exist wherever there are mud crab populations (Le Vay 2001; Barnes *et al.* 2002). Most fishing is artisanal and landings are often not reported (the official catch in 2001 was less than 17,000 tons (FAO, Fishstat 2002)). Exploitation of mud crabs world wide is increasing constantly (Le Vay 2001; Le Vay *et al.* 2001b). In South East Asia where fishing pressure is high, the number and average size of the crabs being caught is declining (Kosuge 2001) and because the fishery is extremely difficult to manage the decline in landings is expected to continue (Le Vay 2001).

Mud crabs are suited to culture because in their post-larval stages they are hardy, tolerant of fluctuations in water quality and temperature, relatively resistant to disease and grow quickly on a wide variety of diets (Williams and Primavera 2001). Records of mud crab aquaculture in China date back more than 100 years (Keenan 1990; Shen and Lai 1994). In its simplest form mud crabs are cultured by fishers in order to add value to poorer quality crabs. Newly-moulted mud crabs have flaccid, watery flesh and thus a low market value and females with immature ovaries are worth less than gravid females. Fishers stock “empty” or “thin” crabs and immature females in bamboo pots, cages or penned enclosures where they are fed trash fish and other “waste” material for 15-40 days until fattened (300-800 g) or ripe (in the case of females) and thus more valuable in the marketplace (Cholik and Hanafi 1992; Dat 1999a; Tan 1999). This is highly profitable and has evolved into more sophisticated farming where wild caught juveniles are bought from fishers and stocked into ponds or enclosures and cultured until they reach market size (Chong 1992; Tan 1999). These forms of aquaculture now form the basis of a widespread industry in South East Asia and over 10,000 tons of farmed mud crab were produced in 2001 (FAO, Fishstat 2002). Mud crabs cannot be farmed intensively because at stocking densities above 1.5 crabs per m<sup>2</sup>, cannibalism significantly reduces harvest (Triño *et al.* 1999). However, their high market value means that culture in separate cages for the soft-shelled market and semi-intensive monoculture or polyculture can also be profitable (Agbayani *et al.* 2001). Staggered and monosex stocking (Triño *et al.* 1999; Triño *et al.* 2001), selective harvesting, provision of shelter - in the form of *Gracilaria* seaweed for example (Triño *et al.* 1999) - and supplementation of the diet with low-cost vegetable and animal feeds (Catacutan *et al.* 2003; Rodríguez *et al.* 2003) have all contributed to better harvests and higher profitability of semi-intensive operations. The development of an artificial diet is being actively researched (Sheen 2000; Triño *et al.* 2001; Catacutan 2002). One of the most exciting prospects for mud crab aquaculture is its potential role in the

reestablishment and conservation of mangrove forests. The crabs benefit from the shelter and primary productivity of mangroves (Triño and Rodríguez 2002). Mud crabs stocked into pens in reforested tidal flats provide rural communities with a sustainable source of income (Triño and Rodríguez 2002) which would otherwise need to come from more destructive utilisation (Barbarosa *et al.* 2001). Increased mangrove habitat results in increased nursery grounds for juvenile crabs and thus a more secure source of wild-caught seed (Agbayani 2001). Mud crab aquaculture can thus be profitable, sustainable and environmentally friendly.

*Scylla serrata* is indigenous to South Africa where the crabs are common in estuaries and bays along the east coast. The southerly limit of the distribution is generally considered to be the Knysna estuary (34 degrees south, 23 degrees east) (Day 1974). The crabs are often caught by recreational fishers, but attempts to introduce a commercial fishery have failed due to very small landings (Robertson 1996). Population density in South African estuaries is low and the crabs are usually widely spread. Figures of one crab per 295 m<sup>2</sup> in the Kleinemonde estuary and one crab per 2700 m<sup>2</sup> in the North lake of St Lucia were recorded by Hill (1979). The local market is not well developed. Small quantities of frozen crab imported (mostly illegally) from neighbouring Mozambique supply the large expatriate Portuguese and Mozambican communities in Johannesburg (Fernando Ribeiro, Instituto Nacional de Investigacao Pesqueira (Fisheries Research Institute), Maputo, Mozambique, pers. comm.). The limited and erratic availability of mud crabs mean that they rarely feature on restaurant menus or seafood distribution catalogues. However mud crabs are popular with coastal communities in South Africa who are familiar with the product and there is excellent potential for developing the local market (especially the growing tourist market) for what is essentially a high quality product (Barnes *et al.* 2002; Fernando Ribeiro pers. comm.). The export potential for mud crab has been recognised for some time (Du Plessis 1971) and quantities of wild-caught mud crab are already being exported to Singapore from Mozambique (Fernando Ribeiro pers. comm.) and illegally from South Africa (Thomas Hecht Department of Ichthyology and Fisheries Science, Rhodes University, Grahamstown, South Africa, pers. comm.).

The initial impetus for developing *Scylla* aquaculture in South Africa came from the private sector. The Liberty Life Education Foundation (LLEF), dedicated to diversifying the South African economy and alleviating poverty through sustainable, novel projects commissioned the Department of Ichthyology and Fisheries Science (DIFS) at Rhodes University to investigate the potential for mud crab farming in the country. A desk-top study revealed that mud crab aquaculture was feasible and held exciting prospects. There are a

number of estuaries along the subtropical north-east coast of South Africa that would be suitable for mud crab farming in pump ashore facilities or pens in mangroves. These sites would be suitable for semi-intensive monoculture, polyculture with fish or high intensity soft shelled crab production. The estuaries would also be suitable for stock enhancement or ranching as mud crabs tend to remain in those estuaries to which they recruit (Hyland *et al.* 1984), which would simplify ownership issues (Rothlisberg *et al.* 1999; Le Vay 2001). South Africa has close ties with neighbouring Mozambique where there are almost 400,000 hectares of mangrove under increasing human pressure. Estuaries and bays along the coast are being deforested at over 1800 hectares per year. The mangroves are mainly used for low income activities such as collecting firewood or are cleared for salt production. (Barbosa *et al.* 2001) and (as discussed earlier), mud crab farming could provide a sustainable alternative. The prospects for mud crab farming in Southern Africa are therefore excellent.

The bottleneck to the expansion of commercial mud crab aquaculture world-wide, is the dependence on wild-caught juveniles for seed (Williams and Primavera 2001). This is both unreliable and unsustainable (Le Vay 2001). Hatchery technology therefore needs to be established before mud crab aquaculture can be developed to its full potential (Camacho and Apya 2001). In South Africa the abundance of juveniles in estuaries is low (Hill 1979; Robertson 1996) and would not be capable of sustaining an industry. Hatchery produced seed is therefore a prerequisite to establishing mud crab aquaculture in the country. Research into the techniques required for mass rearing of mud crab larvae has been intensified in several South East Asian countries over the last decade. At the start of the project in South Africa, very little of the Asian research work had been published. A fact-finding mission to South East Asia was therefore undertaken to Australasia which established that the technology required for mass rearing of the larvae was feasible and imminent. This was confirmed during the attendance of the International Forum on the Culture of Portunid Crabs held in Boracay, The Philippines in December 1998. On the strength of this, it was decided to initiate research in 1999 with a special focus on larval rearing.

A research facility was built on a commercial *Fenneropenaeus indicus* farm, Mtunzini Prawn Farm (MPF) situated on the subtropical north-east coast. The primary motivation for locating the project at MPF was that broodstock were plentiful in the mangrove forests in the adjacent Umlalazi estuary and in the shrimp production ponds. MPF also had several facilities which were available for rent and high quality seawater pumped ashore from a beach-well was available for use. A temperature controlled laboratory scale hatchery was constructed by

welding together two shipping containers. Associated facilities for maturing and spawning broodstock, and producing algae and live food were built.

At the end of 1999, additional funding and support were acquired from the Flemish Inter-university Council (Vl.I.R.) through an own initiative cooperation with the Laboratory of Aquaculture & Artemia Reference Center (ARC) in Ghent, Belgium. The initiative facilitated cooperation with mud crab aquaculture projects in Asia.

### **Specific studies**

Because mud crab aquaculture is new to South Africa (except for Du Plessis' (1971) rearing investigations) it was necessary to investigate several aspects as background to the project. Keenan *et al.*'s (1998) revision of the *Scylla* genus emphasised the importance of a thorough understanding of mud crab taxonomy as a background to fisheries and aquaculture research. Beyond the knowledge that South African mud crabs belong to a single species (*S. serrata*), very little else was known about the population. As pointed out by Keenan (1995) it was thus important to put the research in perspective by determining whether or not hatchery data obtained for mud crab caught in the Umlalazi estuary in Kwazulu-Natal would be representative of the population in the rest of the country. In other words: do South African mud crabs belong to one genetically homogeneous group or do they vary between regions or between estuaries within regions? *S. serrata* were sampled in six South African estuaries, three on the North-East coast (including the Umlalazi), and three on the South-East coast and one in Madagascar for an outlying comparison. Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was used to assess the genetic variation of the populations found in these estuaries. Results of this work are presented in **Chapter 3**.

The first priority in the development of hatchery techniques for any aquaculture species is to ensure a supply of viable eggs (Barnabé 1990). Besides observations by Robertson and Kruger (1994) no information was available on spawning of South African *Scylla serrata* in captivity. Adult crabs were caught from the wild, then matured, spawned, the eggs incubated and hatched. The results of almost 2 years of spawning *Scylla serrata* at MPF are presented in **Chapter 4**. This includes data relating to size at maturity, time in captivity prior to spawning, size of batch, relative fecundity, individual egg mass and size, size of zoea 1 larvae, incubation time and hatch success rate.

Once the larvae had hatched, the first consideration was to identify the optimum first food organism. First feeding is an important event in the life history of most pelagic marine

larvae and has presented bottlenecks to the development of many commercial species (Barnabé 1990). Of the range of live food organisms suitable for marine larvae, rotifers and *Artemia* are by far the most commonly used due to their relative ease of production (Støttrup and McEvoy 2003). Rotifers are usually offered to species which are too small to handle *Artemia* as a first food but *Artemia* are preferred as large quantities of clean, high quality nauplii can be hatched as needed from dry cysts. As opposed to fish, first feeding mud crab larvae are able to tear their prey apart using their maxillules (Craine 1999) and thus do not need to swallow the feed whole. They are also non selective predators, accepting most food particles that they encounter in the water column. This offered an opportunity for introducing *Artemia* and perhaps even inert food items at first feeding. The nutritional balance of inert foods can be manipulated and offer an ideal tool for the investigation of the nutritional requirements of the larvae. Newly hatched South African *S. serrata* larvae have been fed using both *Artemia* and rotifers and had been observed attempting to feed on *Artemia* cysts, but it was not known which represented the optimum first food. The results of experiments which compared rotifers, *Artemia* and hydrated decapsulated cysts as a first food are presented in **Chapter 5**.

It is necessary to wean larvae from their first food onto a larger food item at some stage of development. Although some crab larvae can occasionally be reared on rotifers alone, weaning onto *Artemia* nauplii usually has a positive effect on growth and survival as the heavier, larger nauplii are more substantial food items (Sulkin 1978; Baylon and Failaman 1997; Harvey and Epifanio 1997). The sooner larvae begin feeding on *Artemia* the better, not only from a nutritional perspective, but also because it limits the dependence on rotifer cultures, which are subject to unpredictable crashes (Rombaut 2001). However, introducing *Artemia* nauplii too soon is not ideal. Even when starved, *Artemia* nauplii moult into second instar metanauplii which are sometimes larger than the larvae. Some hatcheries also find it more cost effective to delay the introduction of *Artemia* for as long as possible due to the high cost of the cysts. Experiments were thus conducted where *Artemia* were introduced to the larvae at various times after hatch to determine both how soon the larvae could handle *Artemia* and how late its introduction could be delayed. Results are presented in **Chapter 6**.

A factor contributing to the successful rearing of many “new” mariculture species has been the enrichment of live foods with (n-3) highly unsaturated fatty acids (HUFA) (Watanabe *et al.* 1983; Sorgeloos *et al.* 1987, 1988). Rotifers and *Artemia* are often deficient in (n-3) HUFA and most marine species have a limited ability to manufacture them *de novo*. The effect is that although normal growth and development is often recorded, a lack of HUFA in the diet often leads to mortality when the larvae are stressed. The metamorphosis from zoea 5 to

megalopa is an extremely stressful process for mud crab larvae, manifesting as moult death syndrome (MSD) with high mortalities. In an attempt to reduce these mortalities, live food enriched with different commercial emulsions was fed to the larvae at different stages of development. The results of these experiments are presented in **Chapter 7**.

A review of the literature is normally presented as an introduction to the subject of a thesis. But because mud crab aquaculture is still an emerging industry, particularly the hatchery phase of production, very little peer reviewed literature exists. Research projects have been conducted in China, India, Malaysia, Indonesia, Australia, The Philippines, Vietnam and Japan for at least the last decade, but most of this information has remained in-house, not been published in English (particularly for China and Japan) or has appeared as papers or abstracts in conference proceedings. Du Plessis (1971) published one of the earliest descriptions of *Scylla serrata* larval rearing but since then, interest in mud crab larviculture in South Africa has been sporadic and no further publications have appeared. Because a more applied, empirical approach is commonly taken while establishing new hatchery technology, the most pertinent information often exists as personal observations and unpublished results collected by the various scientists active in the field. This was also the case with our project and after some discussion with scientists from Australia, The Philippines, Belgium, Wales, Vietnam and South Africa it was decided that a collaborative paper which would describe the “state of the art” for the technology was in urgent need. The manuscript (**Chapter 2**) is intended to put the project in perspective and inform the rest of the scientific community.

It is possible that more scientific results and publications could have been generated if a single aspect of mud crab larval biology had been studied. However, as the original impetus for the project came from the private sector, there was a desire to develop practical methods for the mass rearing of mud crab larvae. In addition, in order to rear sufficient larvae on which to conduct specific experiments, a large amount of developmental work had to be done. A variety of systems and zootechnics were developed during the project. Inspiration came from the literature, from the various conferences and field trips and from communication with fellow mud crab larviculturists. A great deal of time, resource and energy was invested in designing, constructing and fine tuning the various systems used for (for example) pre-treatment of seawater, live food culture, egg production and larval rearing. The approach was by necessity empirical and analysable data could thus not be generated. It was felt that describing the evolution of the various systems used was not suitable for inclusion in an academic thesis and will be written up separately in a more technical report. The information provided in chapter 2

and the materials and methods sections of chapters 4-7 will hopefully suffice as a description of the best techniques used for rearing the larvae during the project.

# Chapter 2

## Current status of mud crab (*Scylla* spp.) hatchery technology

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## **Abstract**

A major bottleneck to the expansion of mud crab (*Scylla* spp.) aquaculture is a lack of hatchery produced seed. Although research on egg production and larval rearing techniques has been undertaken for the past 30 years (and intensively for the last decade), little information is available in the primary literature. Most of the technical information has been published in the “grey” literature and in research reports and is largely unavailable to the wider scientific community. This paper attempts to collate and summarise the published and unpublished data together with observations made, and techniques used by scientists currently involved in mud crab hatchery research. The paper describes techniques for broodstock sourcing, maturation, spawning, egg incubation and hatching. Larval rearing techniques are outlined with regard to stocking, water quality requirements, culture systems, feeding techniques and nutrition. Rearing of megalopae to the juvenile stage is also described. Bottlenecks to commercial production are discussed and possible solutions proposed.

## Introduction

The four species of *Scylla*; *S. serrata*, *S. tranquebarica*, *S. paramamosain* and *S. olivacea* are large, primarily carnivorous portunid crabs that are found in coastal and estuarine waters throughout the tropical and subtropical Indo-Pacific region (Keenan *et al.* 1998). Growth is rapid and the life cycle can be closed within a year. The post-larval stages are highly resistant to disease and can tolerate a wide range of environmental conditions. Mud crabs in all their marketed forms have a high value (Agbayani 2001; Wickins and Lee 2002) and are suitable for aquaculture (Williams and Primavera 2001). Currently mudcrab aquaculture is reliant on juveniles or adults caught from the wild (Le Vay 2001). Despite a long history of culture, records of which date back to 1890 (Shen and Lai 1994), world production is low. In 2001 slightly more than 10,000 tons were produced (FAO, Fishstat 2002). The expansion of the industry is currently limited by the lack of hatchery-produced seed (Williams and Primavera 2001).

Research on the breeding of mud crabs started approximately 40 years ago and several early publications describe captive spawning and larval rearing (Ong 1964; Ong 1966; Du Plessis 1971; Brick 1974; Hill 1974). In Japan, research on hatchery production of seed for restocking purposes has been ongoing since 1979 (Fukunaga and Uzumaki 1982; Horiuti and Yamamoto 1987; Hasegawa 1989; Hamasaki 2002). In the last decade there has been a substantial increase in research, fueled in part by the disease problems in the shrimp industry and partly by declining mud crab fisheries in some parts of South East Asia (Le Vay 2001). The results of this research have been presented in workshops and forums in Western Australia (1995), Northern Territories, Australia (1997), The Philippines (1998) and in Vietnam (2001), amongst others. Although some of the results have been published in the primary literature the majority of information on mud crab hatchery technology and aquaculture appears in the “grey” literature and in unpublished research reports. This paper reviews the available literature from all sources, summarises the current hatchery and rearing techniques, identifies research problems and provides comment on current research trends. This will inform those currently involved, or those wishing to become involved in mud crab hatchery production.

## Broodstock

### *Sourcing and maturation*

Broodstock are most commonly sourced from the wild. They are either caught specifically for this purpose (Mann *et al.* 1999a), are bought from markets (Dat 1999a; Quinitio *et al.* 2001) or directly from fishers (Dat 1999a; Marichamy and Rajapackiam 2001). In areas where commercial culture is practiced, mature breeders are sourced from production ponds (Millamena and Quinitio 2000). The life cycle can be closed within 9 months (Quinitio *et al.* 2001).

Mud crabs readily mate in captivity. This occurs while the female is in a soft-shell condition following molting. However, adult females caught from the wild have usually mated (Robertson and Kruger 1994) and carry spermatophores for extended periods, producing viable eggs up to 6 months after capture without the need for mating (Nghia *et al.* 2001a).

Before introducing wild broodstock into the hatchery, the crabs are commonly scrubbed to remove mud, encrusting algae, infestations and detritus. Individual crabs are usually marked for identification by engraving (Millamena and Quinitio 2000; Djunaidah *et al.* 2003; Churchill 2003) or by gluing an identification tag to the shell. They are then disinfected in a strong formalin bath (50-100 mg/L) for periods ranging from 1 hour (Millamena and Bangcaya 2001) to overnight (Mann *et al.* 1999b). Shell disease is commonly observed, especially amongst older crabs that have long intermoult periods (Robertson 1987). The disease seldom causes mortality and regular cleaning of the shell can minimize its effects (Lavilla-Pitogo *et al.* 2001). Various parasites have also been reported, none of which present serious threats to broodstock or egg production (Lavilla-Pitogo *et al.* 2001).

Neither the size nor type of vessel seems to affect maturation or spawning (egg extrusion). Brood crabs have been successfully maintained individually in small bins (60-300 L) or communally in large tanks ranging from 1-12 tons (Williams *et al.* 1998; Dat 1999b; Mann *et al.* 1999a; Millamena and Quinitio 2000; Baylon *et al.* 2001a; Hamasaki 2002). Successful spawning has been achieved at water depths of 25-150 cm (Dat 1999b; Djunaidah *et al.* 2001b; Millamena and Bangcaya 2001). The average stocking densities in maturation systems is approximately 1.5 crabs/m<sup>2</sup> (Mann *et al.* 1999a 1999b) but broodstock can be stocked up to 5/m<sup>2</sup> (Djunaidah *et al.* 2001b). Broodstock are often kept in separate tanks or cages which eliminate the risk of cannibalism and allows for controlled feeding.

Shelters are usually provided to reduce stress and prevent cannibalism (Millamena and Quinitio 2000; Millamena and Bangcaya 2001; Djunaidah *et al.* 2003; Hamasaki 2002). Shelters should be easy to remove so that brood crabs can be observed and the tank cleaned.

Mud crabs require a substratum for spawning. The crab digs a depression in the substratum over which the abdominal flap is extended and into which the eggs are extruded (Rusdi *et al.* 1994). The pleopods then create a current that assists the attachment of eggs to the tertiary setae. Absence of a substratum results in poor egg attachment and potential loss of the batch. Spawning of *S. paramamosain* is improved by providing a mud substratum rather than sand (Djunaidah *et al.* 2001b). It has been observed that the *Zoothamnium* spp. load on the eggs is reduced when the crabs spawn on a mud substrate, which may be a consequence of turbidity (Nguyen Co Thach, Research Institute of Aquaculture III, Nha Trang, Vietnam, pers. comm.). However, sand substratum is more commonly used since it does not affect water quality and allows for the use of under-gravel filters (Churchill 2003). The entire bottom of the tank can be covered with spawning substratum (Williams *et al.* 1998; Millamena and Quinitio 2000). However, it is more common to contain the substratum in a tray (Mann *et al.* 1999a) allowing the rest of the tank to be cleaned more easily. Substratum depths of 50, 80 and 200 mm have been used successfully (Baylon *et al.* 2001a; Djunaidah *et al.* 2001b; Millamena and Bangcaya 2001).

Females also mature their ovaries and extrude eggs in earthen ponds or netted pens. There is some indication in Vietnam that a higher percentage of crabs spawn in ponds, possibly due to reduced stress. The diet available to broodstock in ponds is more varied and probably closer to the one they would encounter in the wild. However, managing broodstock in ponds is more difficult than in tanks and the eggs from broodstock spawned in ponds are occasionally heavily infested with parasites (Quinitio and Parado-Esteva 2003).

In smaller broodstock maturation systems the water is recirculated, while some larger systems operate on a flow-through basis (Marichamy and Rajapackiam 2001; Millamena and Bangcaya 2001; Quinitio *et al.* 2001; Hamasaki 2002) or on partial re-circulation (Williams *et al.* 1998; Mann *et al.* 1999a 1999b; Millamena and Quinitio 2000; Baylon *et al.* 2001a; Millamena and Bangcaya 2001).

Full strength seawater (30-35 g/L) is normally used for captive maturation and spawning of *S. serrata* in the hatchery (Mann *et al.* 1999a). Lower salinities (20-25 g/L) can be used for *S. tranquebarica*, *S. olivacea* and *S. paramamosain* (Ng 1998). However, full strength seawater is still commonly used for maturing and spawning all four species.

Broodstock are generally kept between 25°C and 35°C. A lower critical temperature for egg development of 18°C has been identified (Heasman and Fielder 1983; Hamasaki 2002) but successful ovulation and spawning have been observed at water temperatures ranging from 20-30°C (Mann *et al.* 1999a; Millamena and Bangcaya 2001; Davis *et al.* 2003b). *S. serrata* broodstock extrude significantly larger eggs with higher hatch rates during cooler months (18-22°C) Mann *et al.* (1999a) suggesting that lower temperatures than normal should be applied.

Mud crabs are nocturnal and normally inhabit turbid estuaries (Hill 1978; Dat 1999a; Barnes *et al.* 2002). For this reason broodstock crabs are maintained under low light conditions (Williams *et al.* 1998; Mann *et al.* 1999a; Quintio *et al.* 2001), although darkness is not required for spawning (Nghia *et al.* 2001a). Spawning tanks are normally securely covered in order to prevent the crabs from escaping.

Broodstock are normally fed fresh or frozen foods including penaeid shrimp, squid, fish, bivalves such as clams and mussel, gastropod snails and annelid worms (Dat 1999a; Mann *et al.* 1999a; Millamena and Bangcaya 2001; Quintio *et al.* 2001; Hamasaki 2002). Enriched *Artemia* biomass has also been used as food, presented as agar bound pellets (Djunaidah *et al.* 2003). The natural diet of mud crabs consists mostly of benthic mollusks and crustaceans (Hill 1979) and it is generally agreed that these organisms must be present in the diet. The source and health status of feed organisms, particularly crustaceans, need to be carefully assessed as they can act as vectors for disease as WSSV (Chen *et al.* 2000).

Formulated diets developed for penaeid broodstock have been fed to mud crab broodstock to investigate the effects on fecundity and spawning. Best results have been achieved when formulated feeds have been used as a supplement to fresh or frozen feeds (Millamena and Quintio 2000; Millamena and Bangcaya 2001; Djunaidah *et al.* 2003). Formulated diets are currently not used extensively.

Broodstock is usually fed on an *ad libitum* basis (Mann *et al.* 1999a; Williams *et al.* 1999), but when rationed, crabs are fed at rates ranging from 3-5% (Dat 1999b), 6-10% (Millamena and Bangcaya 2001) to 20% body weighty per day (Baylon and Failaman 2001). Owing to their nocturnal feeding habits (Hill 1978; Barnes *et al.* 2002) food is normally provided in the evening, but feeding twice (Dat 1999a) or three times daily (Millamena and Bangcaya 2001) allows for more careful monitoring of food consumption and better maintenance of water quality.

Li *et al.* (1999) highlighted the importance of (n-6) and (n-3) highly unsaturated fatty acids (HUFA) in broodstock diets. Levels of HUFA and protein in newly hatched larvae can be manipulated by altering the levels in the diet fed to the broodstock (Djunaidah *et al.* 2003). No

studies have yet been undertaken to test the effect of HUFA levels and ratios in broodstock diets on egg quality or larval performance.

### *Spawning (egg extrusion)*

Mud crabs adapt well to artificial conditions and spawn readily in captivity without intervention. Over 85% of wild caught crabs brought into the laboratory or hatchery normally spawn, mostly within 40 days of stocking (Williams *et al.* 1998; Mann *et al.* 1999a; Davis *et al.* 2003b). The status of the ovary can be assessed by biopsy (Mann *et al.* 1999a; Millamena and Bangcaya 2001) or by pushing down on the first abdominal segment which exposes the ovary under the carapace (Djunaidah *et al.* 2003; Quinitio and Parado-Esteva 2003). Mature ovaries are bright yellow to deep orange in colour and fill the body cavity (Quinitio and Parado-Esteva 2003).

Natural spawning is the norm in most hatcheries. However, eyestalk ablation can be used to shorten the pre-spawning period. It is only necessary to ablate one of the eyestalks (Baylon and Failaman 1997; Djunaidah, *et al.* 1998; Dat 1999b; Mann *et al.* 1999a; Millamena and Quinitio 2000; Baylon *et al.* 2001a; Baylon and Failaman 2001; Marichamy and Rajapackiam 2001; Nghia *et al.* 2001a; Quinitio *et al.* 2001). Prior to eyestalk ablation, brood crabs are anaesthetized in an aerated chloroform bath (1-3 g/L). Hot pincers are used to crimp the eyestalk and cauterize the wound. Ablation does not seem to stress the crabs as there is no reduction in survival, fecundity or fertilization rate (Millamena and Bangcaya 2001; Nghia *et al.* 2001b). However, there is little and conflicting information on the effect of ablation on egg and larval quality (Millamena and Quinitio 2000). Mann *et al.* (1999a) reported that ablated crabs produced larger eggs with better hatch rates while Millamena and Bangcaya (2001) recorded lower fertilization and hatch rates of eggs from ablated females (Millamena and Bangcaya 2001).

Captive mud crabs produce viable eggs year round. Some authors have reported seasonal changes and peaks in spawning activity (Mann *et al.* 1999a; Nghia *et al.* 2001a; Marichamy and Rajapackiam 2001; Hamasaki 2002; Davis *et al.* 2003b) especially in areas that have extremes in seasonal water temperatures or salinity (Li *et al.* 1999; Le Vay 2001). However, these are usually not distinct and spawning patterns seem to vary between locations (Hai *et al.* 2001; Nghia *et al.* 2001a). Mud crabs are capable of spawning up to three times from a single mating (Dat 1999a; Marichamy and Rajapackiam 2001; Quinitio *et al.* 2001). Rematuration of the ovary in captive crabs occurs about one month after spawning (Hai *et al.* 2001; Marichamy and

Rajapackiam 2001; Djunaidah *et al.* 2003). Although some authors report no reduction in fecundity or fertilization rate with repeated spawning (Millamena and Qunitio 2000), Dat (1999b) recorded a substantial decrease in egg production. The effect of repeated spawning on egg and larval quality has not been quantified. Female *Scylla serrata* are capable of more than one post pubertal moult (Robertson and Kruger 1994) and spermatophores are retained after molting.

*Scylla* are highly fecund and typically produce more than 1 million eggs per batch. Egg production ranges widely between species, locations and individual crabs. *S. serrata* seems to be more fecund than other *Scylla* species (Srinivasagam *et al.* 2000. Marichamy and Rajapackiam 1992; Jyamanna and Jinadasa 1993; Mann *et al.* 1999a; Bin Jamari 1991; Hai *et al.* 2001; Dat 1999a; Millamena and Bangcaya 2001; Qunitio *et al.* 2001; Djunaidah *et al.* 2001b). Batches of 50,000 are considered small and the largest batch recorded in captivity is 10 million eggs (Davis *et al.* 2003a or b??). Egg number is not a limiting factor to juvenile production. Broodstock matured in captivity are more fecund and produce eggs with a higher rate of fertilization than wild caught crabs. This is apparently due to enhanced environmental conditions and nutrition (Millamena and Bangcaya 2001; Qunitio *et al.* 2001). Broodstock domestication is a high priority area of research.

### *Incubation and hatching*

Brood crabs which have spawned are conspicuous. The large, bright yellow to orange egg mass (“sponge” or “berry”) is carried prominently under the abdominal flap. Berried brood crabs are transferred to incubators as soon as possible after spawning. Flat bottomed glass aquaria of 60 L, plastic containers of 100 L or larger fiberglass tanks of approximately 300-500 L (Mann *et al.* 1999a; Millamena and Qunitio 2000; Millamena and Bangcaya 2001; Hamasaki 2002) and 1000 L (Williams *et al.* 1998) have been used as incubators.

Water in the incubators is usually sterilized with UV light and exchanged either on a flow-through basis or recirculated through biofilters (Williams *et al.* 1998; Mann *et al.* 1999a; Hamasaki 2002). Because of the need for a stable environment during incubation, recirculation is preferred. To maintain water quality and to prevent contamination of the eggs, berried crabs are not fed. Mild aeration is provided and incubators are siphoned clean daily (Mann *et al.* 1999b; Hamasaki 2002).

Poor attachment of the eggs can result in the gradual loss of a brood during incubation. The causes of poorly attached eggs are not well understood, but may include poor initial

attachment of the eggs, poor egg quality, or parasitic and fungal infections (Hai *et al.* 2001; Quinitio *et al.* 2001). When eggs are lost during incubation, the hatch rate of the remaining eggs is generally low (Dat 1999b). Where space is limiting, brood crabs with poorly attached eggs are removed to make space for crabs carrying well attached eggs (Dat 1999b; Churchill 2003; Davis *et al.* 2003a or b??). Dropped eggs can be incubated. However hatch rates are highly variable and fungal infections and eggs adhering to the sides of containers has so far made this practice impractical (Hai *et al.* 2001; Churchill *et al.* 2003).

The eggs can be infected with a variety of parasitic worms, fungus (*Haliphthoros*, *Sirolpidium*, *Atkinsiella* and *Lagenidium* spp.) and ciliates (*Zoothamnium* spp.) (Hamasaki and Hatai 1993a; Churchill 2003). Parasitic infections generally occur if water quality is not adequately maintained and may result in poor embryogenesis or egg loss as a result of brood crabs tearing at the egg mass. The vulnerability of eggs to fungal infection decreases as their development progresses (Hamasaki and Hatai 1993a). Berried brood crabs can be bathed in formalin and/or malachite green as a prophylactic, or can be treated if infestations occur. There is evidence that formalin (25 mg/L) is toxic to eggs up to one day after spawning, although older eggs can tolerate high doses of both formalin (50-150 mg/L) and malachite green (50 mg/L) (Kaji *et al.* 1991; Hamasaki and Hatai 1993b; Churchill 2003; Davis *et al.* 2003). Heavy aeration is applied during the bathing process. Antifungal agents such as Treflan®<sup>1</sup> (44% trifuralin) (0.05 - 0.1 mg/L) or formalin (25 mg/L) can be added to the incubation water resulting in a reduced fungal load of the eggs and preventing transmission of the fungus from the egg surface to the larvae at hatch (Kaji *et al.* 1991; Quinitio *et al.* 2001). Preventative measures, such as maintaining a hygienic incubation environment and good water flow in the tank are also effective and are preferable to anti-microbial chemicals.

At 27-28°C first cleavage occurs 5-8 hours after extrusion (Quinitio and Parado-Esteva 2003). The developing embryo is visible under a dissecting microscope from day 4 after spawning (Djunaidah *et al.* 2003). Embryonic development can occur successfully between 20 and 30°C but temperatures above 26°C are preferred (Li *et al.* 1999; Mann *et al.* 1999a; Quinitio *et al.* 2001). The incubation period is strongly temperature dependent. For *S. serrata*, the relationship is best represented by the equation  $y = 6029.7 - 406.9x + 7.2x^2$  where  $y$  = incubation period and  $x$  = temperature (Churchill 2003) Embryonic development is infinitely protracted below 17°C (Heasman and Fielder 1983). Depending on temperature, mud crab eggs usually hatch within 9-12 days after extrusion (Dat 1999b; Baylon and Failaman 2001; Quinitio

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<sup>1</sup> Mention of a branded product does not mean endorsement by the authors

*et al.* 2001). Time of hatch can be predicted by monitoring development of the embryo. Vigorous limb movement, a beating heart and well developed eye spots with a purplish patch are all indications of imminent hatching. Hatching can also be predicted by measuring egg diameter, which increases during incubation (Mann *et al.* 1999b; Nghia *et al.* 2001a). For *S. serrata* the relationship is best represented by the polynomial equation  $y = 0.299 + 0.002x + 0.001x^2$  where  $y$  = egg diameter and  $x$  = incubation period in days (Churchill 2003).

Hatching usually occurs in the morning (Hai *et al.* 2001; Hamasaki 2002). Brood crabs are often transferred to a separate tank 1-2 days prior to hatching (Hamasaki 2002; Mann *et al.* 1999a 1999b). Hatching tanks tend to be larger than 500 L, providing sufficient space for the hatched larvae to disperse and to delay deterioration of water quality. The water in the hatching tank is usually full strength seawater (30-35 g/L) that has been filtered and treated to reduce the microbial load. A small number of larvae (100s to a few 1000s) normally hatch one day before the main hatch. Hatching is normally a spontaneous and rapid event with 90% of the larvae hatching within 10 minutes. The larvae hatch as pre-zoeae, a non-swimming phase that typically moults into the first zoeal stage within 10 minutes post-hatch. The persistence of pre-zoeae after the normal initial moult time has elapsed is used as an indicator of poor batch quality (Dat 1999b; Mann *et al.* 1999a; Hai *et al.* 2001).

## Larval rearing

### *Selection and stocking*

Once the eggs have hatched, the brood crab is removed from the hatching tank. Hatch success is generally high, ranging from 80-90%. Larvae are negatively buoyant and swim vigorously in order to maintain their position in the water column. In The Philippines, larvae are left in the hatching tank for approximately one hour after hatching, providing sufficient time for “poorer quality” larvae to sink to the bottom so that “better quality” larvae at the surface can be used for rearing (Quinitio and Parado-Esteva 2003). The value of this practice needs to be weighed against the fact that Z1 larvae are highly susceptible to fungal infections both from a variety of sources including the egg envelope (Hamasaki and Hatai 1993a) and in a typical hatching tank of 1 m<sup>3</sup>, bacterial numbers rise rapidly within an hour of hatching (Mann *et al.* 1999b). This suggests that larvae be removed soon after hatching (10-15 minutes). Larvae are sometimes rinsed with clean seawater before transferring them to rearing tanks to reduce the bacterial load (Mann *et al.* 1999b). To transfer the larvae to rearing tanks they are scooped from

the water surface (Baylon and Failaman 1997; Mann *et al.* 1999a; Baylon *et al.* 2001a; Djunaidah *et al.* 2003). Nets cannot be used because the long spines of the larvae become entangled in the mesh and are damaged. Larvae have been reared at densities ranging from 10-200 larvae/L (Djunaidah, *et al.* 1998; Williams *et al.* 1998; Baylon and Failaman 1999; Dat 1999b; Quinitio *et al.* 1999; Quinitio *et al.* 2001) although densities of 30-60/L are more commonly used. No correlation has been found between stocking density and survival although in The Philippines vibriosis has been associated with stocking densities higher than 100 larvae/L.

### *Water quality and parameters*

The larvae require high quality water, free of potential pathogens, predators or parasites. Seawater is commonly filtered to 1 µm and then either chlorinated overnight and dechlorinated with sodium thiosulphate (Parado-Esteva and Quinitio 1998; Williams *et al.* 1998; Mann *et al.* 1999b; Williams *et al.* 1999; Quinitio *et al.* 2001), ozonated and then recirculated through a biofilter, inoculated with nitrifying bacteria and settled for several days before use (Baylon and Failaman 1999; Williams *et al.* 2002) and/or re-filtered through activated carbon and sterilized with UV light (Dat 1999b). Water in the rearing vessels is also allowed to stabilize for several days before introduction of the larvae (Parado-Esteva and Quinitio 1998; Mann *et al.* 1999b) and background algae are sometimes added for apparent antimicrobial qualities. Water pretreatment has been found to significantly improve survival through to megalopa (M) (Mann 1999b; Williams *et al.* 2002).

Early stage *S. serrata* have a lower temperature tolerance of 12°C (Hill 1974). Larvae have been successfully reared at 25-30°C, but temperatures in the upper range (29-30°C) shorten development time (Dat 1999b; Li *et al.* 1999; Quinitio *et al.* 1999; Mann *et al.* 2001; Quinitio *et al.* 2001). Larvae are extremely sensitive to abrupt changes in temperature. Temperatures are maintained within 1°C from hatch to harvest of megalopae. Larvae (particularly during the early stages) are vulnerable to temperature gradients generated by immersion heaters of the type used in shrimp hatcheries (Mann *et al.* 1999b). If temperatures must be increased, low capacity aquarium heaters should be used or heating can be applied indirectly by incubating rearing vessels in heated baths or with remote heaters in the sump of recirculating systems. Heating is unnecessary in large-scale vessels under tropical conditions, as long as diurnal temperature variation is not extreme.

At temperatures above 25°C *S. serrata* Z1 have lower and upper 24 hour salinity LC<sub>50s</sub> of 17.5 g/L (Hill 1974) and 40 g/L respectively (Churchill 2003). *Scylla* Z1-Z4 are usually reared in seawater ranging from 30-35 g/L, depending on locality (Djunaidah *et al.* 1998; Mann *et al.* 2001; Quinitio *et al.* 2001) although in Japan, *S. tranquebarica* are reared at 25 g/L in order to control fungal infections.

Reducing the salinity at Z5 to 20-24 g/L for Z5 *S. tranquebarica* and 20 g/L for *S. olivacea* significantly improves metamorphosis to megalopa (Baylon and Failaman 2001b; Quinitio *et al.* 2001) and reducing salinity from 30 to 25 g/L has been found to trigger metamorphosis of Z5 *S. paramamosain* (Dat 1999b). Seawater is therefore gradually diluted (1-2 g/L daily) at the end of the Z5 stage or at the beginning of the megalopa stage for these three species. *S. serrata* does not seem to require reduced salinity at metamorphosis and the larvae are commonly reared from hatch through to the first crab stage (C1) in full strength seawater (Cowan 1984; Baylon *et al.* 2001b; Mann *et al.* 2001).

Early (Z1 and Z2) larvae are strongly photopositive and light is often used to keep early larvae close to the water surface. The larvae are visual predators, particularly in the latter stages of development (Z3-M), and high light intensities (1800 - 4000 lm/m<sup>2</sup>) are typically applied (Mann *et al.* 2001). Survival and development after the Z3 stage are significantly compromised in the absence of light or under low (50 lm/m<sup>2</sup>) light intensities. Where possible, larvae are reared under natural light (Williams *et al.* 1998; Takeuchi *et al.* 2000).

Larvae seem to require a dark phase (Djunaidah, *et al.* 1998), but no significant effect on survival was recorded between 12 and 18 hour photoperiods (Nghia *et al.* 2001b). Photoperiods of at least 12 hours are generally provided (Mann *et al.* 2001; Quinitio *et al.* 2001).

*S. serrata* larvae can tolerate relatively high levels of nitrogenous waste. A 24 hour LC<sub>50</sub> of 39.7±2.0 mg/L total ammonia nitrogen (TAN) at pH 8.2 was determined for *S. serrata* Z1 in South Africa (Churchill 2003), while in Australia a level of 62 mg/L was determined (Ravi Fotedar, Aquatic Science Research Unit, Muresk Institute, Curtin University, Western Australia, pers. comm.). The Australian study also determined a 24 hour LC<sub>50</sub> for later instars of approximately 50mg/L. Larval growth rate was reduced by 5% after 96 hours in comparison to a control (96 hour EC<sub>5</sub>) at 5-7 mg/L TAN. TAN is generally maintained below 1 mg/L in hatchery runs. *S. serrata* Z1 have a 96 hour LC<sub>50</sub> of 3 mg/L ammonia (NH<sub>3</sub>-N) (Quinitio and Parado-Esteva 2001). All zoeal stages have a 96 hour LC<sub>50</sub> of 80 mg/L nitrite (NO<sub>2</sub><sup>-</sup>-N) (Mary Lyn Seneriches-Abiera, Mindanao State University, General Santos City, The Philippines, pers. comm.). Little research on ammonia tolerance has been conducted for the larvae of the other

species although *S. paramamosain* zoeae survive concentrations of 5 mg/L NH<sub>3</sub>-N in recirculating systems in Vietnam.

No research has been conducted on the tolerance of *Scylla* larvae to extremes in pH and oxygen concentration.

### *Culture systems*

For highly controlled experimental conditions, the vessels used for larval rearing are small, ranging from 100 ml to 5 L in capacity (Zeng 1998; Baylon and Failaman 1999; Quinitio *et al.* 1999; Williams *et al.* 1999; Zeng and Li 1999; Baylon *et al.* 2001a; Mann *et al.* 2001). Highly predictable survival (60-90% up to megalopa) can be achieved in these systems, particularly in the presence of antibiotics.

Larger cylindro-conical, fiberglass tanks are used for investigating zootechnical aspects or for nutritional studies. Under pilot and commercial scale conditions larvae are reared in plastic, fiberglass or reinforced concrete tanks ranging from 1 to 200 m<sup>3</sup> capacity (Fukunaga and Uzumaki 1982; Dat 1999b; Williams *et al.* 1999; Millamena and Bangcaya 2001; Quinitio *et al.* 2001; Hamasaki *et al.* 2002b). Tank colour does not appear to be an important factor in mudcrab larval rearing. A range of tank colours has been used successfully.

Water is exchanged either on a constant flow-through basis, or by draining or siphoning 50-85% of the tank volume daily and replacing it with clean seawater, or by recirculation through a biofilter (100% every 2-3 hours) (Nghia *et al.* 2001b). Under green-water culture conditions water is not exchanged for the first three days. Thereafter, water exchange is slowly increased from 10-20% per day for Z2-Z3 to between 40 and 50% per day at the end of the rearing cycle (Z4-M) (Mann *et al.* 1999b; Quinitio *et al.* 2001). In Japan a mesocosm system is used for culturing larvae in larger tanks (>10 m<sup>3</sup>). The tanks are partially filled with green-water at Z1 (20-25% volume). The tank is filled with clean seawater during the course of the Z2-Z3 stages and during the Z4 and M stages water is exchanged on flow-through basis (Hamasaki *et al.* 2002b).

Dead larvae and uneaten food that accumulate on the tank bottom are generally siphoned out of rearing vessels daily (Quinitio *et al.* 2001; Baylon and Failaman 2001) and care must be taken to avoid siphoning out larvae which have sunk to the bottom of the container. A biofilm develops on the sides of the tank during culture. Williams *et al.* (1998) achieved significantly higher survival in 5 L bowls when the biofilm was removed daily. Cleaning the biofilm from the tank sides can however release large amounts of bacterial flock into the water column and

cleaning must be done by careful vacuuming or the tank must first be drained down before cleaning.

Larvae ingest microalgae by chance when swallowing water. Although the presence of microalgae in the water prolongs the survival of Z1, they cannot moult to Z2 unless the diet is supplemented with zooplankton (Brick 1974). Several genera of microalgae including *Tetraselmis*, *Skeletonema*, *Chlorella*, *Nannochloropsis*, *Chaetoceros* and *Isochrysis* have been added to the rearing water during larval rearing at densities ranging from  $5 \times 10^4$  to  $5 \times 10^5$  cells/ml (Djunaidah, *et al.* 1998; Dat 1999b; Mann *et al.* 1999b; Williams *et al.* 1999; Zeng and Li 1999; Mann *et al.* 2001; Quintio *et al.* 2001) in order to “condition” the water and to serve as food for rotifers and *Artemia*. Species with high HUFA levels such as *N. oculata* and *I. galbana* are added in order to continually enrich rotifers and *Artemia*. The effect of background algae on larval survival and growth is however not clear. The current trend in mud crab larval rearing is to use algae at least during the rotifer feeding stages and for megalopae.

In conclusion, it appears that the current hatchery systems around the world are more a reflection of available facilities (e.g. tank sizes and live food production capacity) than best practice and are indicative that a range of approaches to mud crab larviculture can be successful. An ideal system for mud crab larval rearing has not yet been perfected.

### Feeding and nutrition

Larvae are fed as soon as possible after transfer into the rearing vessels. High protease activity in newly hatched zoeae indicates their ability to digest food immediately after hatch (Li *et al.* 1999). Delaying feeding for up to 24 hours after hatching has no significant effect on survival (Lumasag and Quintio 1998). However the effects of such starvation on the later stages are not known. Starving larvae for longer than 48 hours induces high mortality despite resumption of normal feeding (Lumasag and Quintio 1998; Djunaidah *et al.* 2003). The point of no return (PNR) for newly hatched larvae has been estimated at 30 hours and 96 hours for *S. paramamosain* and *S. serrata*, respectively (Li *et al.* 1998 and 1999). In an earlier study Mann and Parlato (1995) found that *S. serrata* Z1 could survive for up to 142 hours without feeding but were not able to moult to Z2. Temperature significantly influences the PNR. Newly hatched *S. serrata* have a PNR<sub>50</sub> of 57.6 hours at 28°C and 91 hours at 24°C (Lumasag and Quintio 1998).

Z1 and Z2 are usually fed on rotifers (*Brachionus* spp.) (Li *et al.* 1999; Mann *et al.* 1999b; Zeng and Li 1999; Takeuchi *et al.* 2000). Larvae fed rotifers benefit from the

supplementation of *Artemia* nauplii as early as Z1 but Z2 utilize *Artemia* more efficiently (Li *et al.* 1998). If *Artemia* are withheld beyond Z3, growth and survival are compromised (Li *et al.* 1998; Zeng and Li 1999; Takeuchi *et al.* 2000; Suprayadi *et al.* 2002a).

Although larvae can be reared on *Artemia* nauplii from hatch, survival is usually enhanced by the addition of rotifers to the diet (Ong 1966; Baylon and Failaman 1999). Larvae are commonly reared on rotifers during Z1 and Z2 while *Artemia* are usually introduced at Z3 (Li *et al.* 1999; Mann *et al.* 1999b; Takeuchi *et al.* 2000; Nghia *et al.* 2001b; Quinitio *et al.* 2001). However, in large systems (10-100 m<sup>3</sup> tanks) that cannot be flushed regularly, feeding with *Artemia* is delayed to Z4 (Hamasaki *et al.* 2002b). Although *Artemia* are typically provided as newly hatched nauplii, Z3 are large enough to consume metanauplii, allowing for the delivery of supplementary nutrients via bioencapsulation (see below). On-grown *Artemia* (5 days old to adult) provide a larger sized prey item and are fed to Z5 and megalopae (Mann *et al.* 1999b; Quinitio and Parado-Estepa 2003).

Z1 are functionally passive feeders relying on chance encounters with their food (Heasman and Fielder 1983). Laboratory based studies have indicated that high densities of rotifers (30-80/ml) significantly enhance survival (Djunaidah, *et al.* 1998; Zeng and Li 1999; Suprayudi *et al.* 2002a). However, in mass production systems practical considerations such as maintenance of water quality and rotifer production capacity sometimes dictate that lower densities (10-20/ml) are used (Williams *et al.* 1998; Mann *et al.* 1999b; Baylon *et al.* 2001b; Quinitio *et al.* 2001). *Artemia* are generally provided at 0.5-10/ml (Mann *et al.* 1999b; Williams *et al.* 1999; Zeng and Li 1999; Baylon *et al.* 2001a; Mann *et al.* 2001; Quinitio *et al.* 2001; Quinitio *et al.* 2001) although when larvae are densely stocked (100 larvae/L) *Artemia* densities of up to 20/ml are used (Nghia *et al.* 2001b). The optimum density of *Artemia* has not been investigated and it appears that a practical approach is commonly taken with *Artemia* density based on the stocking density of larvae, the culture system used and the available budget.

Mud crab larvae accept inert food. Formulated feeds adapted from shrimp larval diets (Quinitio *et al.* 1999) and dried *Artemia* flakes (Nghia *et al.* 2001b) have been used with some success. Neither is an effective replacement for live food, but they would appear to have some potential as supplements. In the Philippines, it is common practice to supplement live food with 2 mg/L/day formulated feeds in large tanks (>10 m<sup>3</sup>) from Z1 to Z5 (Quinitio *et al.* 2001).

Inferior nutrition may be a factor contributing to the highly variable survival and the high susceptibility to disease often recorded in mud crab larviculture. Poor nutrition, even if confined to the early larval stages, has been suggested as a cause for the phenomenon of moult death syndrome (MDS) - high mortality during or after the moult from Z5 to M (Li *et al.* 1999;

Zeng and Li 1999; Mann *et al.* 2001; Marichamy and Rajapackiam 2001; Quinitio *et al.* 2001; Hamasaki *et al.* 2002a, 2002b; Suprayudi *et al.* 2002b).

It is generally accepted that larvae eat zooplankton in the wild which are nutritionally superior to rotifers and *Artemia*. Copepods commonly caught from the wild such as *Acartia tsuensis* and *Pseudodiaptomus* spp. have been used as live feeds (Toledo *et al.* 1998) but ensuring a regular supply is difficult as they cannot be cultured consistently at high densities (Delbare *et al.* 1996). The nutritional quality of rotifers and *Artemia* can be improved by enriching them with nutrients in a process known as bioencapsulation (Kanazawa and Koshio 1994; Rees *et al.* 1994; Coutteau *et al.* 1997; Wouters *et al.* 1997). The effect of enriching the live food with essential fatty acids (EFA's) contained in algae, yeasts and formulated emulsions on survival and growth of mud crab larvae has been tested.

Suprayudi *et al.* (2002b) recorded significantly improved survival of *S. serrata* larvae after boosting the total (n-3) highly unsaturated fatty acid ( $\Sigma(n-3)$  HUFA) content of rotifers from 3-5 mg/g to 7.6-8 mg/g. However, Hamasaki *et al.* (2002a) reported abnormal development of *S. serrata* Z5 leading to high mortality at metamorphosis to megalopa as a result of feeding the larvae rotifers containing  $\Sigma(n-3)$  HUFA levels above 6 mg/g. Whereas Suprayudi (2002b) recorded high mortality through the moult to megalopa and first crab after feeding rotifers boosted to 31 mg/g  $\Sigma(n-3)$  HUFA, in Vietnam enriching rotifers with emulsions containing 30%  $\Sigma(n-3)$  HUFA enhanced growth of *S. paramamosain* larvae. The contradictory results of these studies indicate that the requirement for  $\Sigma(n-3)$  HUFA may differ between batches or species. They also indicate that specific (n-3) HUFA's may be more important than the absolute levels of (n-3) HUFA.

Suprayudi *et al.* (2002a) found that the low levels (3 mg/g) of eicosapentaenoic acid (20:5 (n-3)) (EPA) and docosahexaenoic acid (22:6 (n-3)) (DHA) (1 mg/g) found in *Artemia* are sufficient for good survival through the moults to M and C1 for *S. serrata*. These results were supported by Mann *et al.* (2001) who found that boosting levels of EPA (39 mg/g) and DHA (15 mg/g) in *Artemia* did not lead to significant improvements in survival. Kobayashi *et al.* (2000) even suggested that levels of 16-35 mg/g EPA and 17-29 mg/g DHA in *Artemia* were excessive, compromising survival of *S. tranquebarica*. However, Kobayashi *et al.* (2000) found that enriching *Artemia* with EPA at 13 mg/g while maintaining DHA at trace levels enhanced survival indicating that the ratio of DHA/EPA in *Artemia* may be more important than absolute levels. There are indications that the DHA/EPA ratio in the rotifers is also important. Kobayashi *et al.* 2000 found that a relatively low DHA/EPA ratio in rotifers (0.07) produced significantly better survival than higher ratios (0.8-0.9) for *S. tranquebarica* larvae. This is supported by

Suprayudi (2002b) who recorded best survival through first metamorphosis for *S. serrata* when feeding rotifers containing a relatively low DHA/EPA ratio of 0.3. However, in Vietnam, zoeal growth and first metamorphosis of *S. paramamosain* larvae were significantly enhanced when the DHA/EPA ratios in the rotifers were high (0.6-4) (Vandendriessche 2003). This is another indication of possible differences in fatty acid requirements between the different *Scylla* species.

High arachidonic acid (ARA) /EPA ratios (0.23) in unenriched as opposed to enriched (0.02-0.05) *Artemia* significantly enhanced larval survival of *S. tranquebarica* (Kobayashi *et al.* 2000), but no other studies on the ARA requirements of the larvae has been conducted.

The results of the experiments conducted on the enrichment of rotifers and *Artemia* with EFA's have generally been contradictory and our understanding of the requirements of larvae for fatty acids is scant. The experiments described above represent work on four different species at different laboratories using different larval rearing techniques. Most of the studies have concentrated on the enrichment of either rotifers or *Artemia*, whereas work in South Africa and Vietnam has revealed that both need to be enriched with EFA's to significantly improve larval performance. It also appears that the larvae may require different quantities and ratios of EFA's at different stages of development. Despite limited information, several inferences concerning the fatty acid requirements of larvae can be made. Firstly, although HUFA rich algae can maintain the nutritional value of previously boosted live food and may supply other essential nutrients, the addition of algae to the rearing tank does not adequately enrich live food with the necessary EFA levels. Live food needs to be artificially boosted before being fed to the larvae. Secondly,  $\Sigma(n-3)$  HUFA levels ranging from 8-10 mg/g in live food may be beneficial, but larvae seem unable to tolerate excessive levels in the diet. Thirdly, EPA and DHA enrichment of the rotifers is required, but the absolute amounts do not seem to be as important as the DHA/EPA ratio.

## Nursery

Cannibalism is a common problem and can account for a large percentage of mortality (30-50%) at both first and second metamorphosis (Dat 1999b; Quinitio *et al.* 2001; Suprayudi *et al.* 2002a). Asynchronous moulting exacerbates the problem (Quinitio *et al.* 2001). Moulting synchronicity can be improved by enriching the live food (Takeuchi *et al.* 1999). Megalopae are commonly transferred to separate rearing systems which reduces the rate of cannibalism. Megalopae change from planktonic to benthic orientation at 4-5 days after first metamorphosis and are typically transferred prior to this change. Metamorphosis to C1 occurs 7-10 days after metamorphosis to M (Baylon and Failaman 1999; Dat 1999b). *S. serrata* megalopae tolerate handling (Quinitio and Parado-Esteva 2000), however megalopae of *S. paramamosain* appear to be more delicate and it is common practice in Vietnam to leave *S. paramamosain* megalopae in the culture tanks until they have moulted to C1.

Megalopae are reared through to crab stages 4-7 (crablet) in flat-bottomed tanks ranging from 8 L (Baylon and Failaman 1999) to 2000-8000 L (Williams *et al.* 1998; Dat 1999b). Cannibalism continues to be a problem throughout the nursery stage though it can be partially averted by placing additional substrata and shelters into the rearing vessels (Marasigan 1998; Mann *et al.* 1999b; Quinitio *et al.* 2001). Coconut palm fronds, seaweeds (e.g. *Gracilaria bailinae*) and a variety of plastic meshes and pipes have been used for this purpose.

Megalopae are also reared in earthen ponds (50 m<sup>2</sup> filled to a depth of 80-100 cm) and prepared to promote a dense plankton bloom prior to stocking (Marasigan 1998; Rodríguez *et al.* 1998). Crablet growth is faster in ponds than in tanks and this is presumably due to the presence of naturally occurring live feeds, although controlling mortality in ponds is more difficult (Rodríguez *et al.* 2001). Management of nursery ponds can be simplified by culturing megalopae and crablets in suspended cages or “hapa” nets (1 mm mesh size and 1x1x1.5 m deep) (Marasigan 1998; Rodríguez *et al.* 2001). Megalopae are stocked at rates of 1 to 150/L in indoor tanks (Baylon and Failaman 1999; Dat 1999b; Quinitio *et al.* 2001) and 0.1/m<sup>2</sup> to 125/m<sup>2</sup> in outdoor ponds (Marasigan 1998; Rodríguez *et al.* 1998). The density at which megalopae are stocked, depends on when the crablets are to be harvested. If crablets are harvested at C1/C2, they can be stocked at densities exceeding 300/m<sup>2</sup>. However, survival to later crab stages is compromised at such high densities (Rodríguez *et al.* 1998).

Megalopae are fed a variety of minced fresh and frozen feeds (Williams *et al.* 1999; Hamasaki *et al.* 2002b; Quinitio and Parado-Esteva 2003) and particulate diets formulated for penaeid prawn post larvae. Supplementation of inert diets with *Artemia* nauplii or adults

significantly increases survival to C1 (Heasman and Fielder 1983; Marasigan 1998; Baylon and Failaman 1999; Mann *et al.* 1999b; Williams *et al.* 1999; Qunitio *et al.* 2001).

### **Bottlenecks to commercial production**

Larvae have been produced on a commercial scale in several countries. Rearing trials conducted on *S. serrata* in China (Li *et al.* 1999) and more recently (2003) in the Philippines and Australia have produced thousands of C1 in commercial shrimp hatcheries with survival ranging from 3.2-10%. In Japan 1.5 million hatchery produced *S. paramamosain* and *S. serrata* juveniles were released in 1999 for restocking purposes (Hamasaki 2002). Although survival rates have improved markedly over the last decade, there are still several bottlenecks that prevent widespread commercial seed production.

Larvae are vulnerable to a variety of diseases and parasites. Under suboptimal conditions, *Zoothamnium*, *Vorticella* and other sessile ciliates can attach to the larval integument (Dat 1999b); fungi of the genera *Haliphthoros*, *Lagedinium* and *Atkinsiella* can be transmitted to newly hatched larvae, which are highly susceptible to infection (Kaji *et al.* 1991; Hamasaki and Hatai 1993a; Qunitio *et al.* 2001). White spot virus (WSSV) (Chen *et al.* 2000) can be introduced.

Bacterial disease is considered to be one of the most important bottlenecks to commercial hatchery production. The best evidence for the role of bacterial pathogens in the hatchery is that regardless of other factors, the only treatment known to significantly reduce mortality is treating the rearing water with antibiotics (Parado-Esteba and Qunitio 1998; Mann 2001). Potential pathogens including *Vibrio* spp. (Mann *et al.* 1999b), luminescent *Vibrio*, filamentous bacteria (Takeuchi *et al.* 2000) and others have been identified in larval cultures where they can rapidly rise to levels generally considered to be deleterious to crustacean larvae. If not controlled, bacterial numbers can increase by 2 log units on successive days (Qunitio *et al.* 2001). Information on how bacteria affect the larvae is limited. Histological studies have thus far not determined the aetiology or mechanism of mortality in mud crab larvae (Mann *et al.* 2001). Additionally there has been no consistent correlation between larval performance and the structure of the bacterial community or the presence or absence of particular bacterial strains. The bacterial community of larval cultures seems to be highly volatile among culture vessels and batches as well as during a culture cycle in a single vessel (Mann *et al.* 2001). Mass cultures do not show the same improved response to hygiene protocols as laboratory scale cultures, possibly due to differing microbial environments. The common thread is that antibiotics

consistently lead to improved production. Although regular application of antibiotics is not a desirable practice for reasons of sustainability, it has frequently been used for experimentation as it is often the only means of ensuring the survival of sufficient larvae from which data can be gathered. Antibiotics are either used as a prophylactic in small scale experiments: e.g. 20 mg/L streptomycin (Takeuchi *et al.* 2000) or 2 mg/L Sodium Nifurstyrenate (Hamasaki *et al.* 2002b) or as a bath (100 mg/L Oxytetracycline) for Z1 larvae before being stocked into pilot scale systems (Baylon and Failaman 2001).

Alternatives to antibiotics such as formalin and probiotic preparations (bacterial strains which inhibit known pathogens) have been applied. Mud crab larvae can tolerate up to 25 mg/L of formalin (Kaji *et al.* 1991) and a 24 hour LC<sub>50</sub> for *S. serrata* Z1 has been estimated at 37 mg/L (Churchill 2003). Regular dosing with 20 mg/L formalin every 2 days is used to prevent fungal (e.g. *Haliphthoros* spp.) and bacterial infection of larvae in Vietnam. Although of less potential risk than antibiotics, microbes can eventually build up resistance to formalin especially if applied at regular, low doses. Results for larval rearing trials using probiotic bacteria (and their products) have been inconclusive thus far. Probiotics have been effective in enhancing seed production for other portunid species (Nogami and Maeda 1992) and the technology is receiving a great deal of attention (Verschuere *et al.* 2000; Irianto and Austin 2002). With continued research, probiotics and immunostimulants may provide good alternatives to antibiotics for mud crab larval rearing in the future (Lavilla-Pitogo *et al.* 2002).

A characteristic of mud crab larval culture is the highly variable survival. Successive batches in a hatchery can produce 80% survival to C1 or total mortality before first metamorphosis. Several authors have suggested that variable survival is caused by differences in egg and larval quality (e.g. Mann *et al.* 1999a; Millamena and Bangcaya 2001). Differences in batch quality may also be a factor contributing to differences in results and apparent contradictions between studies (Zeng and Li 1999). Egg colour has been used to estimate egg quality, but no correlation has been found between initial egg colour and the quality of the eggs or larvae (Churchill 2003). The quality of newly hatched larvae has been tested by subjecting them to stressors such as starvation (Djunaidah *et al.* 2003) or high concentrations of salinity, ammonia and formalin (Churchill 2003). Using stress tests Churchill (2003) identified female size and DHA, EPA and  $\Sigma(n-3)$  content of the eggs as possible determinants of egg and larval quality. Because rearing techniques have not yet been standardized, validating the accuracy of a stress test is difficult. There is also an apparent large variability in larval quality within batches so that practical interpretation of stress test results is difficult. A reliable test for evaluating the quality of Z1 mud crab needs to be developed. Ultimately the domestication of broodstock and

the development of a standardized broodstock diet could reduce variability in larval quality at hatch.

## Discussion

The four *Scylla* species spawn readily in captivity. Millions of eggs with a high hatch rate can be produced, year round from a relatively small number of wild-caught or pond-reared broodstock. Scrupulous hygiene during gonad maturation, egg extrusion and egg incubation is required to reduce infection of the eggs by pathogens which can reduce egg viability and be transmitted to the larvae. The typical high variability in survival may be partly due to variable egg and larval quality. Reliable criteria to determine the quality of newly hatched larvae should be developed. Once the life cycle can be closed reliably, egg and larval quality could be enhanced by providing a domesticated broodstock with a formulated diet.

Mud crab larvae are sensitive to captive conditions and high mortalities at all stages of development are common. Causes of mortality include poor water quality, incorrect or fluctuating environmental conditions, cannibalism, feeding and nutritional deficiencies, parasitic and fungal infections and viral and bacterial disease. In some instances mortality has been reduced during early larval stages, but high mortalities are still commonly recorded towards the end of the rearing period. As the rearing process progresses, water quality deteriorates. Accumulated faeces, uneaten food and dead larvae provide substratum for the proliferation of pathogenic bacteria. Although strict hygiene protocols, pretreatment of seawater and high rates of water exchange can mitigate the problem, antibiotics, formalin and other anti-bacterial prophylactics are still the only way to obtain consistent survival. Manipulation of the bacterial community through the application of probiotics may provide a solution (Lavilla-Pitogo *et al.* 2002). The pathogens associated with mud crab larviculture need to be identified and the mode of infection determined.

Mass mortality at metamorphosis to megalopa and C1 due to MDS has been linked to nutritional deficiencies and excessive levels of antifungal or antibiotic agents. Even early nutritional deficiencies can manifest in MDS at the end of the rearing period. Zoeae can be reared on rotifers and *Artemia* and reducing the bacterial load, optimizing feeding densities and improving nutritional quality through bioencapsulation could reduce MDS and improve resistance to stress and disease (Merchie, *et al.* 1997; Mourente and Rodríguez 1997). A formulated diet could help to identify the specific nutritional requirements of the larvae and aid

the development of immuno-stimulants. A domesticated broodstock could allow genetic selection for resistance to disease (Bachère *et al.* 1995).

Although some research groups have some success in the mass production of mud crab juveniles, there is still much scope for further research and development before seed production for aquaculture becomes economically viable and widely adopted.

# **Chapter 3**

## **Genetic Variation of the Mud crab**

### ***Scylla serrata* (Forskål, 1775)**

### **(Crustacea: Portunidae) in South**

### **African estuaries**

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## Abstract

Populations of *Scylla serrata* (Forskål) were sampled in six South African estuaries and one Madagascan estuary. Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was used to assess the genetic variation of the populations found in these estuaries. A high level of variation with a low geographic bias suggested that the South African population should be considered a single entity. The Madagascan population grouped together as a separate clade from the South African estuarine populations and was therefore probably distinct. The Madagascan population also had a lower level of genetic variation than that found among the populations within South African estuaries. While restocking estuaries by releasing newly hatched larvae would probably not be possible, restocking of estuaries by translocation of cultured juveniles (when hatchery technology becomes available) between South African estuaries may be done with minimal risk of affecting the genetic integrity of the indigenous population of *S. serrata*.

## Introduction

The mud crab genus, *Scylla* (Forskål), is found throughout the Indo-Pacific, with Japan (Tamura 1970), Australia (Heasman and Fielder 1977), Hawaii (Brock 1960) and South Africa (Hill 1975) being boundaries to its distribution. The genus *Scylla* has recently been divided into four separate species: *S. serrata*, *S. olivacea*, *S. tranquebarica* and *S. paramamosain* by Keenan *et al.* (1998). Only *S. serrata* occurs in South Africa (Keenan *et al.* 1998). Adult *S. serrata* are predominantly estuarine and in South Africa are generally restricted to those estuaries into which they recruit as juveniles (Hyland *et al.* 1984). Mating occurs while the crabs are in the estuaries. Ripe females then move offshore to spawn, incubate the eggs and release the hatching larvae (Brick 1974; Hill 1978 and Perrine 1979). The larvae are planktonic, spending on average three weeks as zoea and up to ten days as megalopae before recruiting into estuaries (Brick 1974; Hill 1975).

*Scylla serrata* is one of six species of crab considered the most important on the world market (Wickins and Lee 2002). It can be harvested from the wild for a relatively small capital outlay and is suited to aquaculture and restocking (Le Vay 2001). Commercialization of the species thus represents a potentially lucrative industry (Agbayani 2001). The fishery status of mud crabs in South Africa is, at present, non-commercial (Robertson and Kruger 1994; Wendy Robertson, Oceanographic Research Institute, Durban, South Africa, pers. comm.). Although

the females are highly fecund and can be easily spawned in captivity (Millamena and Bangcaya 2001), difficulties in larval rearing (Camacho and Apya 2001) have restricted commercial aquaculture to a dependence on wild-caught juveniles (Agbayani 2001). Restocking programs have also been limited by this bottleneck (Le Vay 2001).

Two recent studies on *S. serrata* have been carried out to analyze the population structure in genetic terms over the Indo-West Pacific region (Gopurenko *et al.* 1999) and over the Kenya-Zanzibar region on a geographic mesoscale (tens to hundreds of kilometers) (Fratini and Vannini 2002). Some integration of the data from these two studies is possible and this was carried out by Frantini and Vannini (2002). It included South African data in the mesoscale data. However, differences in methodologies between the two studies placed some provisos on the analyses. Gopurenko *et al.* (1999) suggested a dispersal event for *S. serrata* across the Indo-Pacific region in the last one million years with some more recent population isolation event(s). Frantini and Vannini's (2002) results suggest that the South African population is distinct from the Kenya/Zanzibar populations. Gopurenko *et al.* (1999) identified the South African *S. serrata* (eleven individuals) as having only one mitotype which is unique to this group of mud crabs.

One essential question, which has bearing on both conservation of the stock and possible restocking initiatives, concerns the recruitment of juvenile crabs to estuaries: Do they recruit into the same (or neighboring) estuaries from which their parents originated? Alternatively, as little is known about the movement of the females offshore and as the larvae are planktonic, is larval distribution to estuaries relatively random or perhaps based on oceanographic phenomena? To this end, the population structure of mud crab in South African estuaries was studied using molecular tools.

Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Williams *et al.* 1990; Carlson *et al.* 1991; Martin *et al.* 1991) has been effectively used in studies of animal and plant population genetics (Kappe *et al.* 1995; Barker *et al.* 1996). It can be used to study the relationships between individuals within populations and between populations. In this study, the genetic relationships between individuals from various South African estuaries were studied using three selected primers. The study also included samples from Madagascar, whose mud crab population (it has been suggested) is distinct (Gopurenko *et al.* 1999; Frantini and Vannini 2002).

## Materials and Methods

### *Sample sites and sampling*

Six estuaries were sampled on the east coast of South Africa between February and July 1999. In the Eastern Cape province, these were: the Kariega (S 33° 32'; E26° 32'), the Cowie (S33° 31'; E26° 46') and the Kei (S32° 30'; E28° 18') and in northern Kwazulu-Natal, these were: the Umlalazi (S28° 55'; E31° 45'), Richards Bay (Mhlatuze) (S28° 45'; E32° 50') and Kosi Bay (S26° 53'; E31° 45') (Fig. 1). The estuary of Morandava (S31° 20'; E44° 25') is on the Madagascan west coast and was used as an outlying group.

Ten crabs were collected from each estuary with the exception of the Kei where only eight crabs were obtained. They were trapped using hoop nets and individually stored in 70% ethanol.

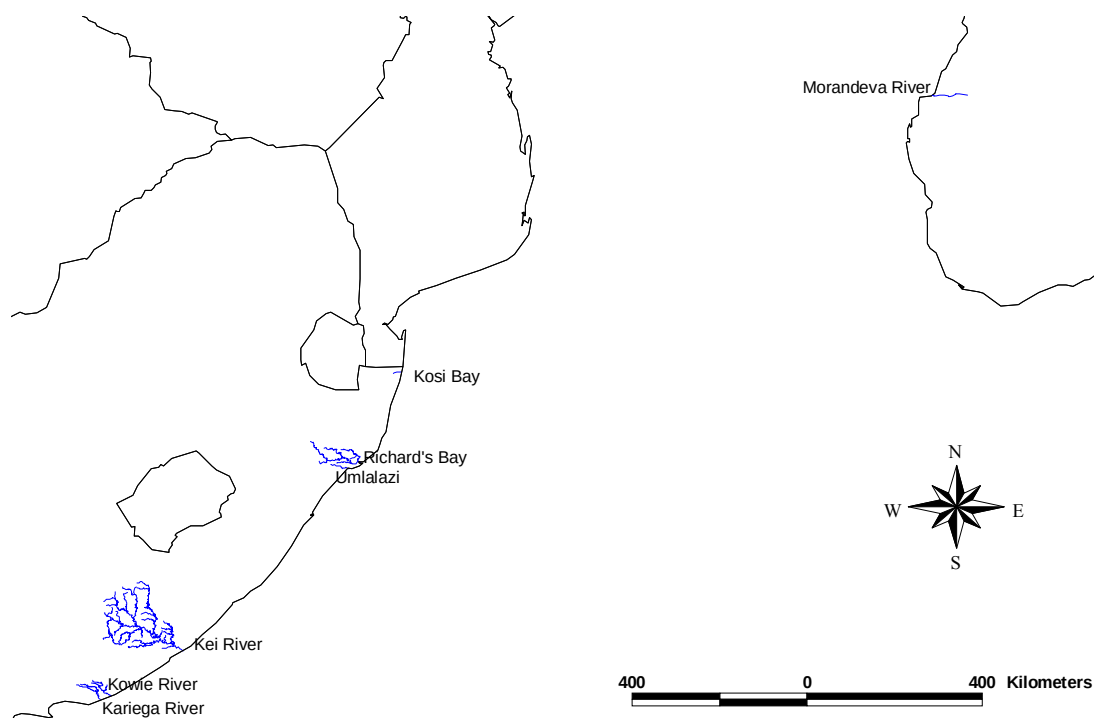


**Fig. 1.** Map of the southern African east coast including south Madagascar showing the sample sites.

### *DNA extraction*

Muscle tissue (25 g) from the walking leg was shredded using a sterile scalpel and the DNA extracted using a High Pure PCR Template Preparation Kit (Boehringer, Mannheim) using the method recommended for tissue. The DNA was eluted in 500  $\mu\text{L}$  of Tris-HCL/EDTA buffer pH 7.2 and stored at 4°C .

### *RAPD-PCR*

The 50 $\mu\text{L}$  reaction consisted of: 2 $\mu\text{L}$  DNA, 1 unit of Taq polymerase (Promega), 100  $\mu\text{M}$  dNTPs, 100  $\mu\text{M}$  primer and 2.4  $\mu\text{M}$   $\text{MgCl}_2$  .

The following conditions were used in a Hybaid thermal cycler: 1 x 95°C for 2 min; 30 x 96°C for 30 sec, 38°C for 30 sec, 72°C for 80 sec); 1 x 72°C for 3min.

Three of sixteen primers tested produced good, consistent RAPD-PCR bands and polymorphisms: RP1 ACTGTACAGT; RP2 GCAAGTAGCT; and RP11 GCAAGTAGTG.

Samples that failed to amplify after two attempts were excluded.

### *Electrophoresis*

Separation was carried out on 2% agarose gels containing ethidium bromide with randomized loading of samples from different estuaries. The RAPD-PCR profiles were captured using a UVP Gel Documentation System 2000, stored in TIFF format and processed using Gel-Compar.

### *Analysis of genetic variation*

The DNA polymorphisms produced by the three primers were analyzed separately and then pooled into a single genetic polymorphism matrix. This matrix was then analyzed using Mix (PHYLIP 3.5c), RAPDPLOT (wcb@lamar.colostate.edu) and NEIGHBOUR (PHYLIP 3.5c), Gene Diversity/M values/Slatkins linearized  $F_{ST}$  (Arlequin ver. 1.1) and analysis of molecular variance AMOVA (Arlequin ver 1.1). The phylogenetic analysis is presented as an unrooted phenogram by MIX. Similar phenograms were obtained by the other methods.

## Results

Only five out of the ten Madagascan samples amplified whereas 55 out of 58 South African samples amplified. The phenogram representing genetic relationships among individual crabs for the pooled primer data using MIX is presented in Fig. 2. The results show the average gene diversity over the RAPD-PCR loci as measured by Arlequin for the various sample sites with Morandava showing by far the lowest level of gene diversity (Table 1).

**Table 1.** Average genetic diversity over the RAPD-PCR loci.

|              |             |
|--------------|-------------|
| Kareiga      | 0.323±0.180 |
| Kei          | 0.278±0.163 |
| Kowie        | 0.257±0.143 |
| Kosi Bay     | 0.330±0.184 |
| Richards Bay | 0.249±0.141 |
| Mtunzini     | 0.251±0.139 |
| Morandava    | 0.073±0.052 |

Table 2 presents a matrix of the Slatkin linearized  $F_{ST}$  (which is an index of population diversity assuming no migration) for the seven sites. This indicates that the populations at the South African sites show only limited divergence from each other but that the Morandava population has diverged from the South African populations.

**Table 2.** Matrix of Slatkin linearised  $F_{ST}$

|              | Kariega | Kei  | Kowie | Kosi Bay | Richards Bay | Mtunzini |
|--------------|---------|------|-------|----------|--------------|----------|
| Kariega      | -       |      |       |          |              |          |
| Kei          | 0.00    | -    |       |          |              |          |
| Kowie        | 0.02    | 0.06 | -     |          |              |          |
| Kosi Bay     | 0.11    | 0.11 | 0.10  | -        |              |          |
| Richards Bay | 0.00    | 0.04 | 0.02  | 0.04     | -            |          |
| Mtunzini     | 0.00    | 0.06 | 0.05  | 0.0      | 0.0          | -        |
| Morandava    | 0.42    | 0.72 | 0.71  | 0.29     | 0.43         | 0.42     |

The matrix of  $M$  values (migration rate assuming no mutation) for the same sites is shown in Table 3.

**Table 3.** Matrix of  $M$  values ( $M = (1 - F_{ST})/(2 F_{ST})$ )

|              | Kariega | Kei   | Kowie | Kosi Bay | Richards Bay | Mtunzini |
|--------------|---------|-------|-------|----------|--------------|----------|
| Kariega      | -       |       |       |          |              |          |
| Kei          | Inf     | -     |       |          |              |          |
| Kowie        | 32.36   | 8.23  | -     |          |              |          |
| Kosi Bay     | 49.50   | 4.69  | 5.26  | -        |              |          |
| Richards Bay | Inf     | 13.87 | 27.83 | 13.70    | -            |          |
| Mtunzini     | Inf     | 8.50  | 9.17  | Inf      | Inf          | -        |
| Morandava    | 1.19    | 0.70  | 0.70  | 1.82     | 1.15         | 1.20     |

An analysis of molecular variance for the individuals, the populations and the groups of estuaries (with most of the variation being found within the populations) is shown in Table 4. Two structures were tested: Madagascan against South African estuaries and Madagascan against Kwazulu-Natal against Eastern Cape Estuaries. Neither was supported statistically at the 5% level although the former gave the highest  $P$  value. Inter-estuary and intra-estuary values were very low.

**Table 4.** Analysis of molecular variance for individuals, estuaries and groups of estuaries using Arlequin 1.1.

| Source of variation                                         | d.f. | Variance components | % Variation | Significance (922 permutations) |
|-------------------------------------------------------------|------|---------------------|-------------|---------------------------------|
| A) Structure. South African estuaries vs Madagascan estuary |      |                     |             |                                 |
| Among groups                                                | 1    | 2.17887             | 23.43       | $0.15323 \pm 0.01144^*$         |
| Among Populations within Groups                             | 5    | 0.24588             | 2.64        | $0.2722 \pm 0.0056^*$           |
| Within Populations                                          | 52   | 6.87277             | 73.92       | $0.0000 \pm 0.0000^*$           |
| Total                                                       | 58   | 430.915             |             |                                 |

**Table 4.** Continued

B) Structure. Eastern Cape estuaries vs Kwazulu-Natal estuaries vs Madagascan estuary

| Source of variation             | d.f. | Variance components | % Variation | Significance (922 permutations) |
|---------------------------------|------|---------------------|-------------|---------------------------------|
| Among groups                    | 2    | 0.73956             | 9.53        | 0.02520 ± 0.00511*              |
| Among Populations within Groups | 4    | 0.14481             | 1.87        | 0.06754 ± 0.00918*              |
| Within Populations              | 52   | 6.87277             | 88.60       | 0.0000 ± 0.0000*                |
| Total                           | 58   | 430.915             |             |                                 |

\*Not significant at 5% level.

Of the three primers used, RP1 generated the largest degree of polymorphism, RP2 slightly less and RP11 the least. Comparisons both with the individual primers (data not shown) and the pooled database showed that the Madagascan samples grouped tightly together with a distinctly lower overall genetic diversity compared to the South African estuaries. The South African samples showed no obvious major geographic clustering between crabs from various estuaries as they were scattered throughout the phenogram. It should be noted, however, that in the phenogram individuals from the same estuary did occasionally cluster together, for example Kei Estuary 1, Kei Estuary 4 and Kei Estuary 7 (Group A); Cowie4. Cowie Estuary 6 and Cowie Estuary 8 (Group B) and Umlalazi Estuary 8 and Umlalazi Estuary 10 (Group C).

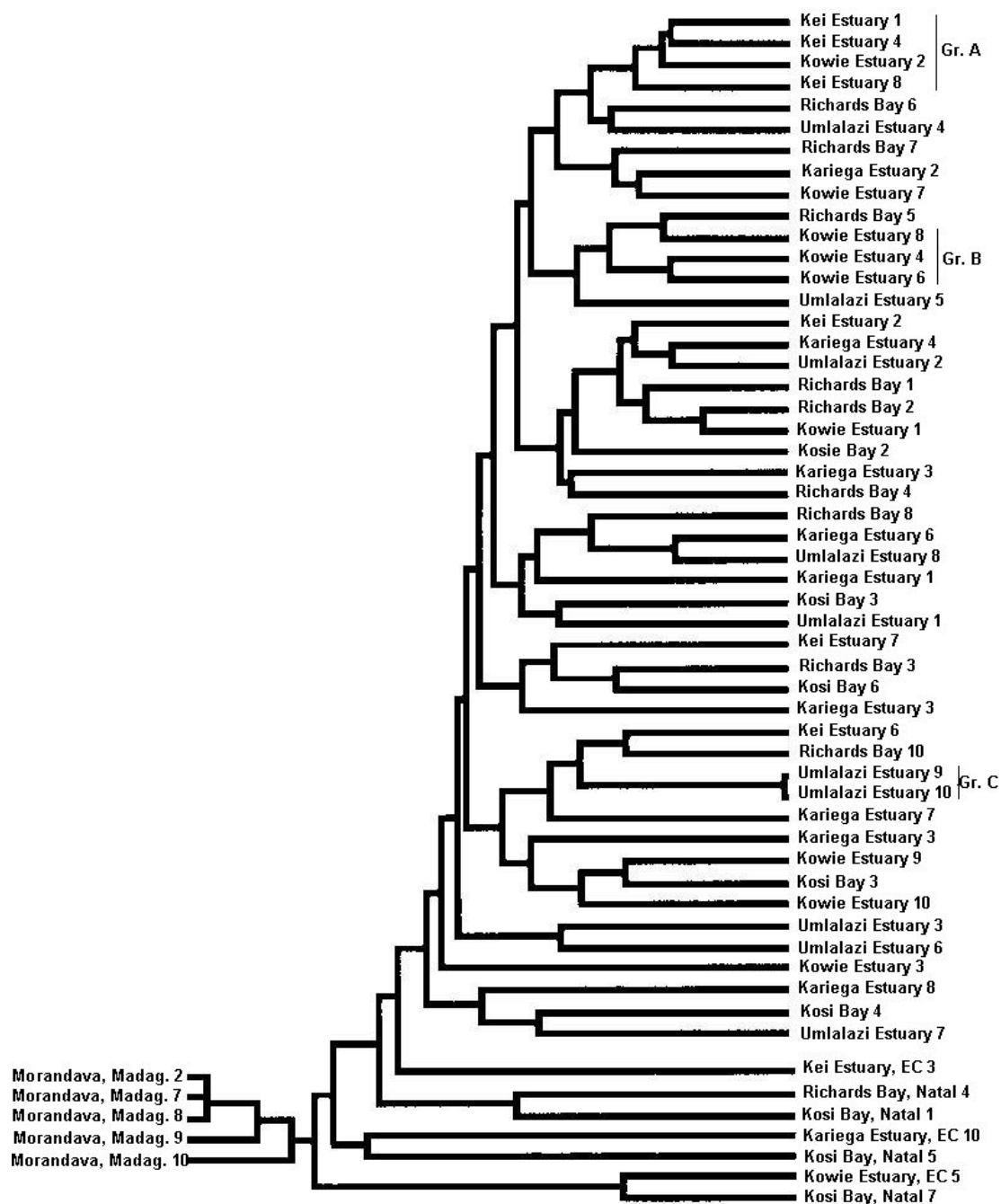


**Fig 2.** Phenogram produced using pooled RAPD-PCR data and MIX. Samples are indicated by the names of the estuary from which they were sampled and a number. This is an unrooted tree. EC = Eastern Cape; Madag. = Madagascar; Gr. = Group.

## Discussion

Mark release and recapture studies of mud crabs have shown that adults of *S. serrata* move little between estuaries separated by regions of unsuitable habitat (Perrine 1979; Hyland 1984). The contribution of adult migration to gene flow between South African estuaries cannot, therefore, be very high. Table 3 suggests that migration could be important between South African populations of mud crabs, but random recruitment to individual estuaries from a common larval population could have the same effect on *M* values as migration. Thus although migration may play a small part, genetic variation within an estuary must be largely a function of recruitment as well as the overall genetic variation of the population. If a low number of crabs colonize an estuary, it is possible that a founder effect could occur but this cannot be true for large populations. At one extreme, if the larvae, which enter an estuary to form the next generation of adult crabs, were limited to those larvae produced by parents from that estuary, this would result in individual estuaries becoming genetically distinct. At the other extreme, if larval recruitment to each estuary were random and from a single mixed larval pool, then no geographic distinction in the genetic profile of each estuary would be detectable. In this case, regional populations should be distinguishable from other regional populations where the regional larval pools do not mix entirely. Such regional pools could exist separately for the Eastern Cape, Northern Kwazulu-Natal and Madagascar. A third possibility exists between these extremes, where there is a regional larval pool from which some local recruitment takes place. This would result in limited geographic structuring.

The data presented by Gopurenko *et al.* (1999) indicates that the South African mud crab population consists of a single mitochondrial haplotype found in no other population of mud crabs. However, the TGGE method used may have been insensitive to variation at some nucleotide positions. With this proviso, Fratini and Vannini's (2002) results support the hypothesis that the South African population is genetically distinct. The presence of silent variation within the South African haplotype would, of necessity, still have to be unique to South African mud crabs. Such cryptic variation would, however, mean that the population had a higher genetic diversity than Gopurenko *et al.*'s (1999) results would suggest.

The phenogram shows that individuals from South African estuaries show only limited genetic relatedness. Samples from the same estuary do not group together, which would be expected if offspring were recruited back to the parental estuary. The wide dispersal across the phenogram of the individuals from a single geographic location and the lack of correlation of genotype with geography would seem to best fit a model whereby all estuaries recruit their

mud crabs from a mixed regional larval pool. The lack of population subdivision found in this study neither supports nor contradicts the possibility of the South African mud crab population bearing a single or limited mitotype. What the results do show is that a recent bottleneck is unlikely because of the high intrapopulation genetic diversity and that if the mitochondrial variation of South African mud crabs is limited, this is rather due to a founder effect. The results also support gene flow over the length of the South African Indian Ocean coast.

The results presented here also support a genetic separation of the South African and Madagascan mud crab populations as suggested by Fratini and Vannini (2002). The phenogram presented in Fig. 2 is unrooted and as the Madagascan branch contains no South African individuals and vice versa, this is evidence supporting a very low level of gene flow (Table 3). Thus, as the Madagascan mud crabs do group together in the phenogram quite tightly, this suggests that they come from a different recruitment pool. They also show a lower genetic diversity suggesting that the recruitment pool is smaller with limited genetic diversity. The migration offshore of ripe females before release of their offspring has been well documented (Hill 1978; Heasman and Fielder 1983). When this, together with the dispersal of the pelagic larval stages by ocean currents was taken into account, it was suggested by Heasman and Fielder (1983) that the *S. serrata* off the east coast of Australia be regarded as a single stock. This was later supported by Gopurenko *et al.* (1999). The same may hold for the east coast mud crabs of South Africa. Although the sample size of the Madagascan crabs was small, the fact that they form a single clade agrees with Fratini and Vannini (2002) and therefore could be attributed to geographic distance and a distinct larval population.

The data for the Morandava crab population describe a single small sample from one estuary in Madagascar. While this sample would seem to be genetically distinct from the South African samples and shows a lower genetic diversity, in order to completely elucidate the genetic relationships of *S. serrata* along the Indian Ocean coast of Africa more complete sampling and analysis using both mitochondrial and nuclear markers needs to be done. However, with the data from Gopurenko *et al.* (1999) and Fratini and Vannini (2002), it would seem that distance, population size, the Agulhas Current as a barrier and biological factors could all play a part in the creation of the above differences. It is even possible that there may be differentiation at the subspecies level along the east African coast in a similar manner to northern Australian mud crabs.

The average gene diversity of the mud crab populations within the various South African estuaries is very much higher than that of the Madagascan population. This most probably indicates a relatively large genetically diverse population although the maintenance

of this diversity by balanced selection cannot be excluded. However, RAPD-PCR markers are inherently randomly selected from the genome and in part are unlikely to be under any form of selection. The M values in Table 3 support the hypothesis that, because of offshore recruitment from a common gene pool, there is a relatively high gene flow between estuaries (assuming mutation plays little part). The Slatkin linearized  $F_{ST}$  values of Table 2 show that the populations in South African estuaries have diverged very little from each other. Thus they either form a single genetic population or have recently diverged from each other after isolation. Because females which have been fertilized within an estuary move offshore to spawn and release the hatching larvae, there is mixing of the populations of hatched larvae from different estuaries. This supports the former hypothesis. A single South African genetic population is also supported by the AMOVA results (Table 4) where the majority of variance is found among individuals within populations (88.6%); variance between estuaries contributed only 1.87% and between groups of estuaries (Morandava, Kwazulu-Kwazulu-Natal and Eastern Cape) only 9.53%. The Madagascan/South African population split is not supported statistically, probably because of the limited size of the Madagascan sample. The fact that this study only used one type of genetic marker system, RAPDs, is limiting and further studies using mitochondrial genotypes on a large scale as well as other chromosomal marker systems are needed to further support any conclusions. However, it is probable that there is a single genetic pool of individuals from which estuarine mud crabs are recruited on the east coast of South Africa which represents a single stock from the view of commercial exploitation. There would, to use an extreme example, be no major apparent influence on the gene pool if crabs from a single estuary were fished to commercial extinction.

The major oceanographic feature of the South African larval recruitment area of *S. serrata* is the Agulhas Current, which flows parallel to the continental shelf off the east coast of South Africa at a speed greater than 1m/s in a southerly direction (Grundlingh 1983, Goshen and Schumann 1990). This has been suggested as a method of southwards dispersal of early life stages for many species (Heydorn *et al.* 1978; Van Der Elst 1981; Beckley and Ballegooyen 1992). If this were the sole mechanism of distributing the larval stages of *S. serrata*, a geographic cline of genetic markers would be expected from Kwazulu-Natal southwards with the Kwazulu-Natal population not recruiting from those further south and thus becoming genetically distinct from the Eastern Cape population. It would also be expected that the genetic diversity of the Eastern Cape population would be higher than that of the Kwazulu-Natal population. A similar effect might occur from estuary to estuary depending on the rate at which individuals flowed south. The Eastern Cape and Kwazulu-Natal estuary groups are not

genetically distinct, the former does not have a significantly higher genetic diversity and thus this cline does not seem to occur as expected. The presence of inshore counter-currents on the continental shelf itself could allow northward recruitment to occur. Thus, although it is clear that the east coast specimens of *S. serrata* represent a single population, these results do not shed light on the actual mechanism of mud crab larval distribution or recruitment, except to suggest that they are not solely dependent on the Agulhas Current.

### **Acknowledgements**

This research would not have been possible without the assistance of the following people and organisations: Wendy Robertson, late of the Oceanographic Research Institute in Durban for sharing her vast mudcrab knowledge; KZN Wildlife, East Cape Nature Conservation and Dr Colin Buxton for collecting samples from the wild, and all those who sat in boats all weekend only to give up their crab dinners for science. The financial assistance from the Liberty Life Foundation through Hylton Appelbaum and the Rhodes University Joint Research Committee for Financial Support facilitated the research.



# Chapter 4

## Spawning Characteristics of the South African Mudcrab *Scylla serrata* (Forskål) in captivity

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### Abstract

*Scylla serrata* is a potential aquaculture species in Southern Africa. Information about its reproductive biology is required as a prerequisite to establishing hatchery technology. Adult female *Scylla serrata* were caught in the Umlalazi estuary on the subtropical east coast of South Africa and kept in captivity to observe and record spawning characteristics. Data collected included crab size and mass, time in captivity prior to spawning, fecundity per batch, relative fecundity, individual egg mass and size, size of zoea 1 larvae, incubation time and hatch success rate. Of the 119 crabs kept in captivity 83 % spawned in the maturation system - most within 40 days of capture. The crabs were highly fecund (mean relative fecundity per batch =  $10,655 \pm 4,069$  eggs/g female) and the majority of the batches hatched within 288 hours (12 days) at 27°C. Spawning in captivity occurred throughout the year, with a peak in late winter/early spring. This differs slightly from records of ovarian maturity stages of the crabs in the wild. A pattern of synchronous spawning was recorded where the females were observed to extrude their eggs in groups, commonly within 3 days of one another, separated by long periods of inactivity, suggesting an exogenous spawning cue. A total of 1 374 488 zoea larvae were obtained per kg of female per month. This means that if sufficient mature females can be caught from the wild, these could be used for stocking hatchery operations. The crabs were easy to maintain, mature and spawn in captivity. This will facilitate future domestication which will eventually reduce the need for wild caught broodstock. The spawning characteristics of South African *Scylla serrata* fit in well with those observed for the genus throughout its distribution implying that ecological and fisheries management could be similar.

### Introduction

*Scylla serrata* is a large, commercially important, brackish water, carnivorous crab, distributed throughout the Indo-Pacific region (Brock 1960; Tamura 1970; Hill 1975; Heasman and Fielder 1977). The species is a candidate for aquaculture in Southern Africa, but a major bottleneck to the establishment of mud crab farming world wide is the dependence on wild caught juveniles (Williams and Primavera 2001). Hatchery techniques thus need to be developed for the species in the region. An essential component of hatchery production is the establishment of a broodstock which can produce a predictable supply of fertilised eggs (Wickins and Lee 2002). A thorough understanding of the reproductive biology of the species is thus required. Despite a renewed research effort worldwide (Wickins and Lee 2002), driven

in part by dwindling natural fisheries for this species (Le Vay 2001), little has been published in the primary literature about the spawning characteristics of *S. serrata*. In addition, much of the work that was published before Keenan *et al.*'s (1998) revision of the genus leaves some doubt as to the precise species from which data were collected.

South African mudcrabs are known to mate and undergo ovarian maturation in estuaries (Hill 1975 1994; Robertson and Kruger 1994). They then migrate offshore to spawn on the continental shelf. Fertilised eggs attach to the pleopods where they are incubated. Hatched larvae are released into the ocean (Hill 1975; Heasman and Fielder 1983; Hill 1994; Le Vay 2001). Berried females are caught sporadically in offshore trawl nets and are extremely rare in estuaries. Observations on spawning behaviour and data of egg production and hatching are therefore scant. The only information on reproduction of *Scylla serrata* in South Africa are seasonal ovarian maturation indices of crabs in the wild and some anecdotal data on spawning in captivity (Robertson and Kruger 1994).

The aim of the research was to gather information about the spawning characteristics of wild-caught *Scylla serrata* in captivity. This information will be used as a basis for husbandry practice and to establish hatchery techniques which will contribute to the eventual domestication of the species. The information can also be used as a component for conserving the wild population or managing the fishery (Caddy 1989; Koslow 1992). In order to achieve this, mature females were caught from the wild and maintained in our research facility on the subtropical east coast of South Africa (Rhodes University Mudcrab Hatchery at Mtunzini Prawn Farm).

## **Materials and Methods**

### *Broodstock Capture*

Mature female crabs were caught from February 2000 to August 2001 using hoop-and-bag traps in the Umlalazi estuary (31°47'E; 28°57'S) on the sub-tropical east coast of South Africa. The traps were not size selective and mature males and immature females were returned to the water immediately. As the Umlalazi estuary is a proclaimed estuarine sanctuary we were also required to return the mature females unharmed to this estuary on completion of our observations. Water temperatures in the estuaries ranged from 17 to 22°C in winter and from 22 to 30°C in summer. Females were identified as being sexually mature by their wide, dark, U-shaped abdomens fringed with setae (Warner 1977; Heasman 1980; Robertson and

Kruger 1994; Ruppert and Barnes 1994). Fishing was conducted only during daylight hours. Traps were checked at 15 minute intervals and the crabs were removed and transported to our facility in a 200 L plastic bin covered with a wet hessian sack. Catch per unit effort (CPUE) was calculated for mature females as:  $(nc/t)/nt$  where:  $nc$ =number of crabs caught;  $t$ =soak time in hours;  $nt$ =number of traps, and expressed as crabs per trap hour (Robertson 1989).

The animals were disinfected against bacteria and fungi by bathing them overnight in an aerated seawater bath containing 100 mg/L malachite green and 100 mg/L formalin (Castille and Addison 1986; Hamasaki and Hatai 1993). Size was recorded (carapace width in mm and weight in grams), and each crab was numbered by engraving the carapace using a jeweller's engraver.

### *Broodstock Conditioning*

The crabs were maintained in a recirculating system at a density not exceeding 1.5 animals/m<sup>2</sup>. No male crabs were introduced into the spawning system as the females were presumed to have mated in the wild and thus be carrying spermatophores (Robertson and Kruger 1994). The 12 m<sup>3</sup> maturation tank (4 x 3 x 1m) had a concrete bottom that was painted black and water depth was maintained at 1 m. Nine round plastic bins (40 cm in diameter, 15 cm in height) filled with beach sand were placed on the bottom of the tank to facilitate spawning. Twelve shelters in the form of roof tiles raised on plastic pipes were placed on the bottom of the tank to reduce cannibalism and stress. Water was recirculated through a 600 L biological trickle filter at a rate of 1 L/sec. Prior to reaching the trickling filter the water was passed through a nylon stocking to collect particulate waste. The stocking was cleaned daily and replaced once a month. Approximately 1 m<sup>3</sup> of the water was replaced daily with fresh seawater at a salinity of 30-32 mg/L, pumped from a beach well point and filtered to 1 µm. Light was subdued (1-3 lm/m<sup>2</sup>, Lutron LX – 105 light meter, Taiwan) by painting the fluorescent lamp covers green and photoperiod was maintained at a constant 14L: 10D cycle. The system was indoors but temperature was not controlled, and varied from 19°C in winter to 25°C in summer. Crabs were fed to satiation on a feeding tray, twice per day with squid (*Loligo* sp.), shrimp (*Fenneropenaeus indicus*), mussels (*Perna perna*) or fish roe (assorted species) on successive days. Uneaten food was removed daily and the tank bottom was vacuumed once a week.

### *Spawning, Incubation and Hatching*

Crabs that spawned (extruded eggs) were easily detected by shining a torch into the tank and turning over the artificial shelters. Berried females were netted out of the maturation tank as soon as they were observed and placed into 60 L glass observation aquaria provided with an external 15 L airlift biological sand filter. The aquaria were filled with seawater from the main recirculating system and once the crabs had been introduced, the water was heated with a 150 watt submerged aquarium heater to 27°C over 24 hours. The crabs were not fed after extruding their eggs, but the aquaria were siphoned daily to remove faeces and dropped eggs. Dropped eggs were examined under a microscope and were commonly found to be infected with fungi, polychaet or nematode worms. Females were then treated with 100 mg/L malachite green and 100 mg/L formalin for 24 hours. If after treatment the female continued to drop large quantities of eggs she was considered unsuitable for further work and immediately returned to the estuary of origin.

Once a berried crab was judged to be a suitable hatchery candidate it was moved to a covered 600 L fibreglass, cylindro-conical incubation tank. A window was provided in the side of the tank for observing the female. The tank was filled with filtered (1 µm), UV sterilised seawater. The water was recirculated through an 80 L biological airlift filter and then passed through a UV steriliser at a rate of 1.7 L/min. Water was drained from the tank into the filter through a 0.3 mm outlet screen to prevent loss of hatching zoea. A sheet of perforated PVC placed at the bottom of the cylindrical portion of the tank provided the crabs with a flat substratum that allowed dropped eggs and faeces to sink into the conical tank bottom from where they could be removed by opening a valve.

Wild-caught crabs were added to the main maturation tank to maintain the stocking density after crabs that had spawned had been removed. Once hatching had taken place, the crabs were returned to their estuary of origin.

### *Measurement of parameters*

Fecundity in the text refers to the numbers of eggs extruded per female per batch and was estimated as follows: berried crabs were weighed shortly after the extrusion of eggs and reweighed after the larvae had hatched. This gave the total mass of the eggs. Four crabs starved for the 12 day incubation lost  $1.14 \pm 0.80\%$  of their body weight. This was considered negligible and was therefore not included in further calculations. Total egg mass was calculated as:

Total egg mass = mass of crab at extrusion – mass of crab after hatch.

Sub-samples of at least 300 eggs were removed from the egg bolus as soon as possible after extrusion using sterilised forceps, weighed and counted in three replicates under a dissecting microscope. This provided an estimate of individual egg mass, whereupon fecundity was estimated by dividing total egg mass by individual egg mass. To validate this method the total number of eggs produced by two females was counted and compared to these estimates.

Egg diameter and larval size (distance between the lateral spine tips) were measured under a compound microscope using a graduated eyepiece at 40 times magnification.

Hatching success was calculated as follows: after hatching in the 600 L tank the larvae were mixed well by vigorous aeration. Nine 50 ml samples were scooped out of the tank at different depths. The larvae in each sample were counted and an average was calculated as the density of larvae per 50 ml, allowing for the estimation of the number of larvae in the 600 L tank. Hatching success (%) was calculated by:

$$(\text{Number of larvae hatched} / \text{Number of eggs extruded}) \times 100.$$

### *Statistics*

A regression model was fitted to the data to determine the relationship between variables using SPSS 11.0.1 (2001, SPSS, Belgium) software. Data was then checked for normality, linearity, homoscedacity and a residual plot was done. Pearson's coefficient was used to examine correlation between variates. Linear, Quadratic Cubic and Power curve estimates were used to determine best fit (Sokal and Rohlf 1995). Data are presented as mean  $\pm$  standard deviation.

## **Results**

### *Female broodstock capture*

Monthly catch per unit effort (CPUE) was not related to season. A total of 119 crabs were caught. CPUE ranged from 0.05 in July and August to 0.16 mature female crabs per trap hour in March and June (Fig. 1). The monthly CPUE averaged over the 19 month sampling period was  $0.10 \pm 0.04$  mature female crabs per trap hour. No traps were deployed in October 2000 due to extreme weather conditions.

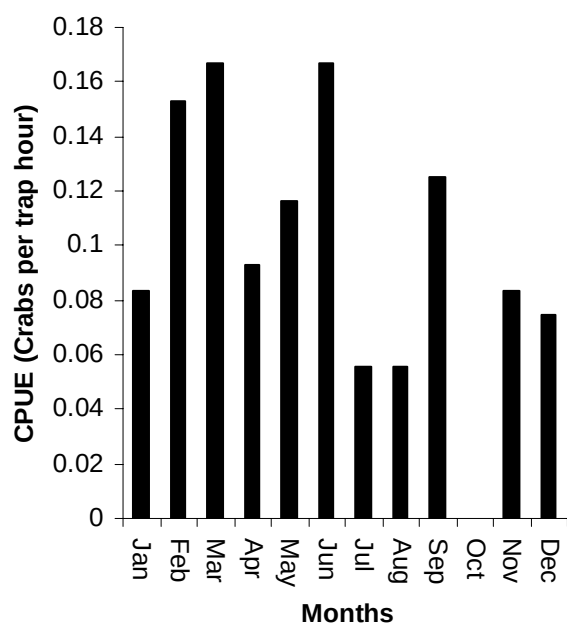


**Figure 1.** Monthly catch per unit effort averaged from data collected over a 19-month period for mature female *Scylla serrata* caught in the sub tropical Umlalazi estuary, South Africa (n=119).

Carapace width (CW) of mature females ranged between 90-200 mm, with 91 % of the females being larger than 120 mm and smaller than 170 mm CW (Fig. 2).

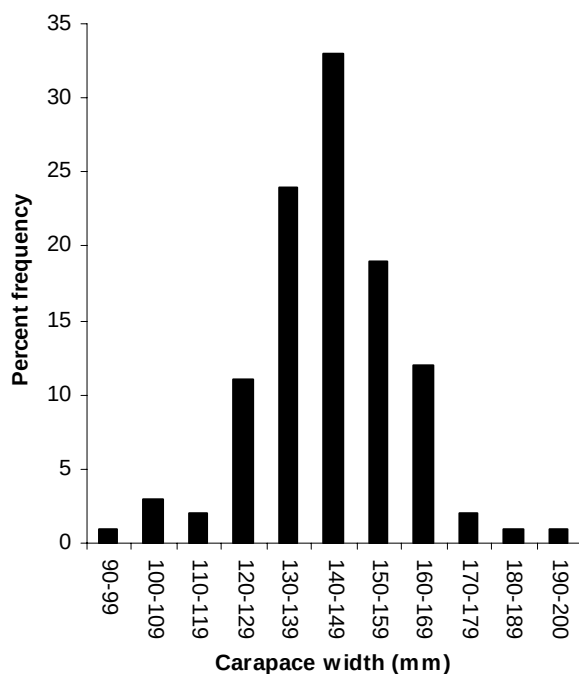


**Figure 2.** Size frequency distribution of mature female *Scylla serrata* caught in the sub tropical Umlalazi estuary, South Africa (n= 119).

## Spawning Characteristics

There was a highly significant correlation ( $P = 0.0001$ ) between CW and mass. The relationship was best expressed as  $\text{Mass} = 0.0014 \cdot \text{CW}^{2.56}$  ( $n = 119$ , Pearson's correlation coefficient = 0.91) (Fig. 3).

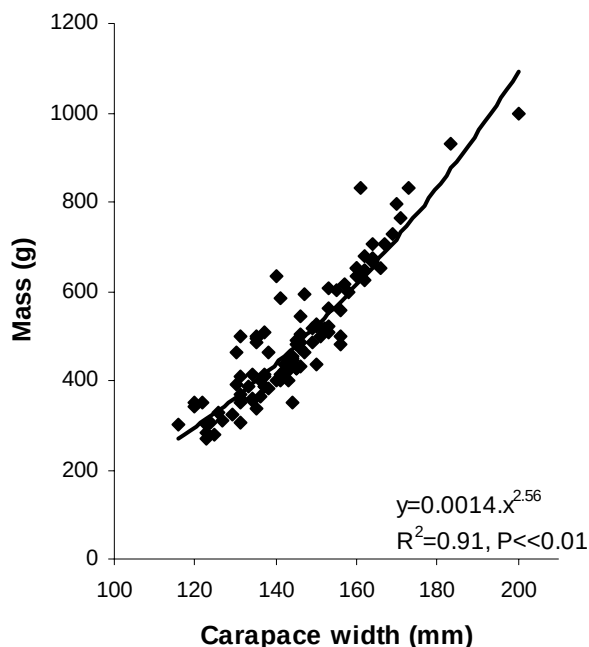


**Figure 3.** Relationship between carapace width and mass for mature female *Scylla serrata* caught in the sub tropical Umlalazi estuary, South Africa ( $n=119$ ).

## Spawning Characteristics

Table 1 summarises the spawning parameters of the crabs that spawned over the 19-month period.

Of the 119 crabs stocked into the system 98 (82.6%) spawned. Cannibalism of newly moulted crabs was the only cause of mortality. Only 4 crabs were lost as a result of this as low moulting frequency amongst mature crabs and high rates of feeding reduced cannibalism to very low levels.

**Table 1.** Spawning characteristics of 98 female *Scylla serrata* spawned over a 19 month period in Kwazulu Natal, South Africa.

| Parameter                                          | Minimum | Median | Maximum | Mean (Std. Dev.) | n  |
|----------------------------------------------------|---------|--------|---------|------------------|----|
| CW of crabs which spawned (mm)                     | 116     | 146    | 200     | 145 (14)         | 69 |
| Mass of crabs which spawned (g)                    | 270     | 464    | 1000    | 490 (144)        | 69 |
| Time to spawning (days in captivity)               | 5       | 31     | 112     | 38 (23)          | 69 |
| Fecundity: (millions of eggs/female/batch)         | 2.45    | 4.73   | 10.75   | 5.17 (1.93)      | 28 |
| Relative fecundity (no. of eggs/g of female/batch) | 3 825   | 9 866  | 25 503  | 10 655 (4 069)   | 28 |
| Total egg mass (g of eggs/female/batch)            | 50      | 101    | 250     | 109.40 (37.45)   | 28 |
| Calculated mass of individual eggs ( $\mu$ g)      | 16.23   | 21.85  | 31.78   | 22.00 (3.91)     | 28 |
| Egg diameter at extrusion (mm)                     | 0.29    | 0.30   | 0.33    | 0.30 (0.01)      | 28 |
| Egg diameter – just prior to hatch (mm)            | 0.38    | 0.40   | 0.41    | 0.40 (0.01)      | 28 |
| Incubation time (hours at 27°C )                   | 233     | 288    | 335     | 288 (21)         | 18 |
| Percent hatching                                   | 72      | 84     | 97      | 84 (6)           | 28 |
| Width of Z1between lateral carapace spines (mm)    | 0.75    | 0.81   | 0.87    | 0.81 (0.03)      | 10 |

Berried crabs did not bury themselves in the sand of the spawning bins and were often observed sitting on top of the shelters or swimming in the water column. The majority of the crabs (62%) spawned within 40 days of being stocked into the system. There was no relationship between time to spawn (days in captivity) and season of the year. Fecundity was significantly correlated ( $p = 0.026$ ) to carapace width, although this correlation was weak ( $R^2 = 0.18$ ) as some smaller crabs produced large batches and some large crabs produced small batches of eggs (Fig. 4).

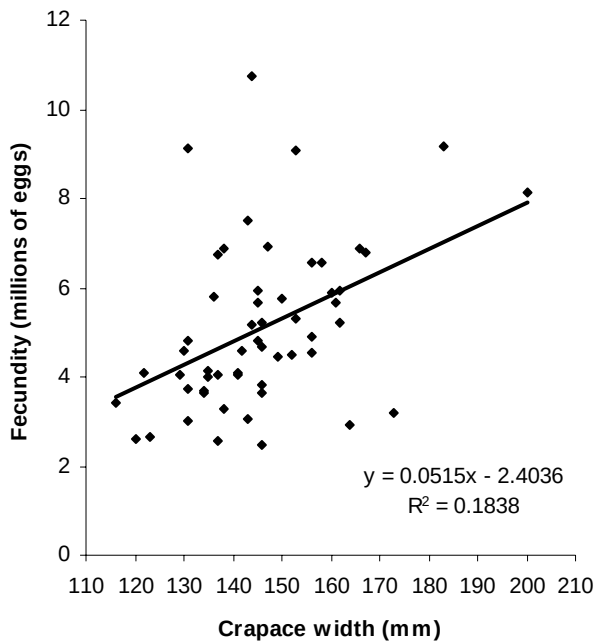


**Figure 4.** Relationship between carapace width (mm) and fecundity (number of eggs per female per batch) for *Scylla serrata* ( $R^2=0.184$ ;  $p=0.026$ ;  $n=60$ ).

The difference between the calculated estimates of egg number and actual egg number of the two females was found to be less than 2% ( $\pm 100,000$  eggs). Average fecundity was  $5.17 \pm 1.93$  million eggs per mature female, within the size range 116 to 200 mm CW. Mean relative fecundity was  $10\,655 \pm 4\,069$  eggs/gram of female. The mean mass of individual eggs was  $22.00 \pm 3.91$   $\mu\text{g}$  ( $n=28$ ).

During the incubation period the eggs increased in size from  $0.30 \pm 0.01$  mm at extrusion to  $0.39 \pm 0.01$  mm just prior to hatching ( $n=28$ ) (Fig. 5).

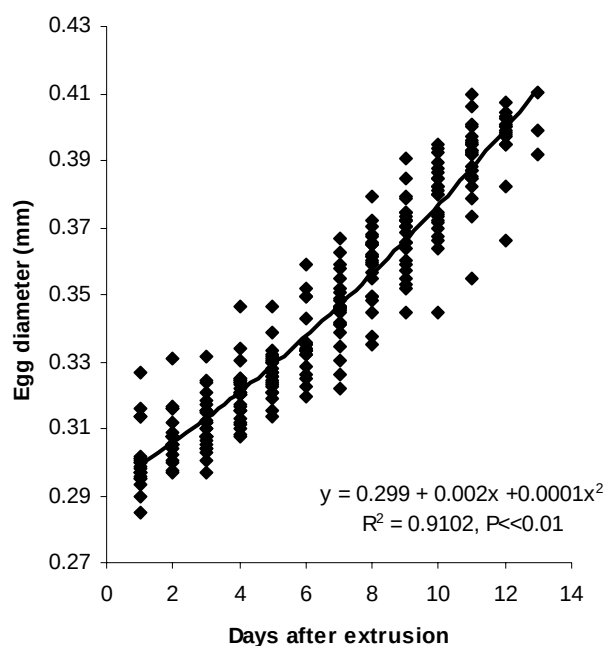


**Figure 5.** Relationship between incubation time (hours) and egg diameter at 27°C for *Scylla serrata* (n=37).

The mean incubation period from extrusion to hatching at a constant temperature of 27°C was  $288 \pm 21$  hours (n = 37). There was a significant correlation between egg diameter and incubation time at 27°C ( $p < 0.001$ ). The relationship was best represented by the polynomial equation  $y = 0.299 + 0.002x + 0.0001x^2$ , where y = days from extrusion and x = egg diameter in mm. This allows for the prediction of time to hatch depending on egg size, at 27°C. There was little variation in the size of the zoea larvae at hatching (mean  $0.81 \pm 0.03$  mm, range 0.75 – 0.87 mm, n=10). Mean hatch rate (proportion of Z1 larvae hatching from extruded eggs) was  $84 \pm 6\%$  (n=28).

All berried females lost a proportion of their eggs during the incubation period, but the amount varied considerably between individuals: the mass of eggs dropped per batch ranged from 0.6 to 22.0 %, (average =  $6.5 \pm 6.0$  %, n=28) of the total mass of eggs extruded. Dropped eggs were examined under a microscope and  $85 \pm 5\%$  were fertilised but were found to be infested with fungus and polychaet worms. In most cases eggs were lost as a result of females grooming the bolus.

Spawning Patterns

The crabs extruded eggs during all months of the year, with a peak during July and August (Fig. 6).

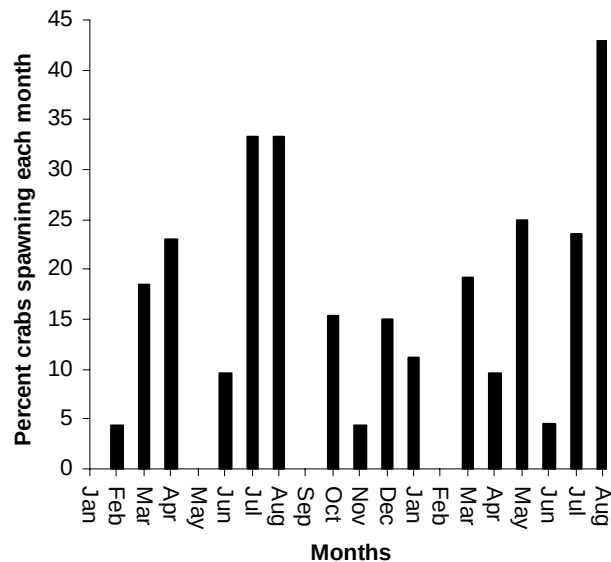


Figure 6. Spawning periodicity of captive *Scylla serrata* (n=98).

Most crabs were observed to spawn within a few days of one another, separated by periods ranging from 10 to 41 days during which no crabs spawned (Fig. 7).

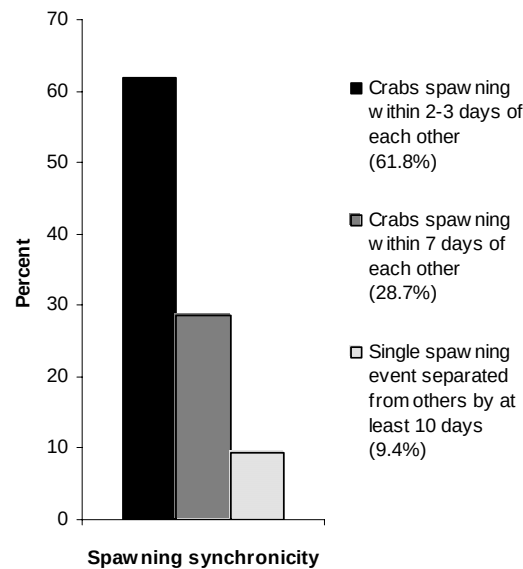


Figure 7. Group spawning pattern of captive female *Scylla serrata* over 19 months (n=98).

## Discussion

### *Sexual maturity and catch data*

Catch per unit effort was lowest during the winter months of July and August: figures for these months were less than half those recorded in March and June and lower than the average yearly CPUE (Fig. 1). This coincided with the period during which the highest proportion of females spawned in the laboratory (Fig. 6). Female crabs with ripe ovaries leave estuaries in order to extrude their eggs at sea (Heasman and Fielder 1983; Hill 1994; Le Vay 2001). Low CPUE during these months could therefore have been a consequence of a lower relative abundance of mature females in the estuaries. Similar reasoning was used by Mann *et al.* (1999a) to explain lower than expected Gonado-Somatic Index (GSI) values of mature females caught in Moreton Bay (Australia) during the period corresponding to high spawning activity of the crabs in captivity. More data are, however, required to substantiate this suggestion particularly as Robertson (1989) suggested that CPUE is a poor index of abundance. While the CPUE of mature females was relatively low, it was consistent and an adequate number of crabs were caught to provide a regular supply of broodstock for hatchery purposes.

Although crabs as small as 90 mm CW were identified as mature according to their external morphology, the smallest crab which spawned in the system was 116 mm CW, thus corroborating suggestions by Ong (1966), Marichamy and Rajapackiam (1992) and Robertson and Kruger (1994) that female *S. serrata* do not necessarily attain true functional maturity at the same size they attain morphological maturity. For practical hatchery purposes, where space is a limiting factor, only crabs larger than 115 mm CW should be used.

### *Fecundity*

Fecundity in most crab genera generally increases with size (Prasad and Neelakantan 1989; Norman and Jones 1993; Haddon 1994; Lardies and Wehrtmann 1996; Mantelatto and Fransozo 1997). Although this trend was observed for mudcrabs in this study, high variability within size classes resulted in a poor correlation between CW and fecundity. Mudcrabs are known to be able to fertilize more than one batch of eggs (usually 2 to 3) from a single mating (Ong 1966; Heasman *et al.* 1985; Millamena and Quintio 2000; Djunaidah *et al.* 2001b). As is the case for other brachyurans (Gardner 1997), it has been found that fecundity in *S. serrata*

usually decreases with each subsequent batch of eggs (Ong 1966). Since females were used for only a single spawning in this study, the smaller egg mass produced by some of the larger females may have been the second or third batch from a single moult and mating, while large batches produced by some of the smaller females may have been a first spawning. This may explain the poor correlation between crab size and batch size. Table 2 lists observed fecundity data of *Scylla* species. The number of eggs per batch and relative fecundity of South African crabs was high compared to the range recorded in Australasia (Bin Jamari 1992; Marichamy and Rajapackiam 1992; Jyamanna and Jinadasa 1993; Dat 1999; Mann *et al.* 1999a; Srinivasagam *et al.* 2000; Djunaidah *et al.* 2001b; Hai *et al.* 2001; Millamena and Bangcaya 2001; Qunitio *et al.* 2001).

**Table 2.** Fecundity and reproductive output of female *Scylla* species from different locations.

| Species                                                | Crab size:<br>CW (mm)<br>mass (g) | Fecundity<br>(avg. number of eggs/<br>female/batch x 10 <sup>6</sup> ) | Reproductive output<br>(number of eggs/batch/g<br>female) | Geographical<br>location | Reference                       |
|--------------------------------------------------------|-----------------------------------|------------------------------------------------------------------------|-----------------------------------------------------------|--------------------------|---------------------------------|
| <i>Scylla</i> sp. (described as <i>tranquebarica</i> ) | 129-175<br>300-690                | 2.1-4.0                                                                | 153-433                                                   | India                    | Srinivasagam <i>et al.</i> 2000 |
| <i>Scylla</i> sp. (described as <i>serrata</i> )       | 85-137<br>98-340                  | 0.1-2.3                                                                | 168-265                                                   | India                    | Srinivasagam <i>et al.</i> 2000 |
| <i>Scylla</i> sp                                       | 117-140<br>N/A                    | 1.5-2                                                                  | N/A                                                       | India                    | Marichamy and Rajapackiam 1992  |
| <i>S. serrata</i>                                      | N/A<br>N/A                        | 1.5-3                                                                  | N/A                                                       | Sri Lanka                | Jyamanna and Jinadasa 1993      |
| <i>S. serrata</i>                                      | 164-170<br>744-820                | 0.4-8.4                                                                | 543-11,531                                                | Australia                | Mann <i>et al.</i> 1999a        |
| <i>Scylla</i> (described as <i>serrata</i> )           | N/A<br>350-520                    | 0.8 - 2                                                                | N/A                                                       | Malaysia                 | Bin Jamari 1992                 |
| <i>S. paramamosain</i>                                 | N/A<br>200-300                    | 0.6 – 3.0                                                              | N/A                                                       | Vietnam                  | Hai <i>et al.</i> 2001          |
| <i>S. paramamosain</i>                                 | 69-169<br>170-790                 | 0.05-1.8 (ZI larvae)                                                   | N/A                                                       | Vietnam                  | Dat 1999                        |
| <i>S. serrata</i>                                      | N/A<br>350-400                    | 1.2-1.6                                                                | 2,319-5,262                                               | The Philippines          | Millamena and Bangcaya 2001     |
| <i>S. serrata</i>                                      | 130-174<br>313-950                | 0.4 – 5.2 (ZI larvae)                                                  | N/A                                                       | The Philippines          | Quinitio <i>et al.</i> 2001     |
| <i>Scylla</i> sp.                                      | N/A<br>200-300                    | 2.7-3.3                                                                | N/A                                                       | Indonesia                | Djunaidah <i>et al.</i> 2001b   |
| <i>S. serrata</i>                                      | 120-170<br>270-1000               | 2.5-10.8                                                               | 3,825-25,503                                              | South Africa             | This study                      |

### *Seasonality of Spawning*

Patterns of seasonal reproductive activity of mud crabs worldwide were reviewed by Le Vay (2001). Maturation and spawning of *Scylla* both in the wild and in captivity has been observed year-round. There are some seasonal peaks, though these differ according to climate and latitude. In this study the peak spawning period in captivity, over the 19 month period was in late winter (July and August). This coincided with the seasonal rise in sea surface temperature and mirrored the peak in spawning for *Sesarma meinerti*, another large estuarine crab in the region (Emmerson 1994). This did not, however, fit well with the incidence of mature ovaries in February, May and October found by Robertson and Kruger (1994) for the same population, or their suggestion that spawning probably occurred from late spring to early autumn. It should however be noted that Robertson and Kruger (1994) recorded high variability in GSI values. This gives substance to the suggestion by Heasman *et al.* (1985) that GSI values were not a good indicator of spawning periodicity for mudcrab. The true situation could, however, have been confused by the fact that the animals in this study were brought into artificial conditions that may have provided cues different to those that the crabs might have experienced in the wild. Mudcrabs are known to be able to maintain ovarian maturity for extended periods before spawning (Heasman *et al.* 1985). The introduction of the females to captive conditions may have induced them to spawn earlier than in the wild. In contrast to the studies by Mann *et al.* (1999a) and Nghia *et al.* (2001a), who found a strong correlation between the time taken to spawn in captivity and season, we made no such observations. Only long-term studies of spawning and recruitment will clear up the discrepancy of whether mudcrabs in South Africa have a distinct spawning season.

### *Spawning patterns*

Spawning synchronicity is common amongst crustaceans (Olive 1992) and is often associated with food abundance, resulting in optimum egg quality. It also ensures that larvae encounter optimal conditions for growth (photoperiod and temperature) and favourable dispersion of the larvae (currents) (Olive 1992; Emmerson 1994; Olive 1995; Greco and Rodríguez 1999).

Although spawning synchronicity of *Scylla serrata* in captivity (Fig. 7) has not been previously documented, it has been observed in Australia. Pheromones have been shown to affect sexual behaviour in crabs (Gleeson 1991). Evidence also exists that hatching eggs release hormones, which influence female crab behaviour (Gleeson and Smith 1984; Kamio *et*

*al.* 2002; Tankersley *et al.* 2002). Despite a paucity of evidence in the literature it is possible that pheromones could be released during spawning that could induce other females to spawn. Researchers in the Philippines have, however, not recorded synchronous spawning in mudcrabs kept in the same system. Also, attempts to induce maturation or spawning with exogenous hormones in decapods such as shrimp, for example, have been largely unsuccessful (Chamberlain 1985). There are two other possible explanations for this phenomenon. Firstly, that changes in conditions in the broodstock maturation system acted as cues to spawning, but were so subtle as to go undetected and secondly, that mudcrabs caught in the wild have moulted, mated and matured their ovaries with some degree of synchrony. This synchrony was then carried through the captive maturation process, resulting in synchronous spawning. There is also some evidence that the lunar cycle coordinates egg laying and hatching in the lobster *Homarus gammarus*, which has benefits for the larvae (Ferrero *et al.* 2002) although Hai *et al.* (2001) found that spawning in *Scylla* did not follow moon phases but no such correlation was recorded in this study.

There were no obvious correlations between spawning patterns and parameters measured in the broodstock system during this study. If, however, spawning is synchronised by a naturally occurring cue, it could be a useful tool for broodstock management and would be a worthwhile area of research. Whatever the mechanisms behind this phenomenon, the results implied that when using wild-caught females, more than one batch of eggs was usually available for experimental or rearing purposes.

#### *Other Aspects of Spawning*

As most of the crabs spawned soon after introduction to the maturation system, it was unnecessary to ablate the eyestalk to induce maturation and spawning – a relatively common practice for mudcrabs (Mann *et al.* 1999a; Millamena and Quinitio 2000; Millamena and Bangcaya 2001). The maturation tank size and stocking densities used in this study allowed for an adequate number of crabs to be held in captivity to ensure a relatively constant supply of viable eggs.

We observed that when berried females could not access the spawning bins due to larger territorial females occupying them, the eggs did not attach well to the pleopods and resulted in a complete loss of the batch. When enough bins were provided, however, the beach sand they contained proved an ideal substrate, negating the need for the (more difficult to maintain) mud-based medium used by Djunaidah *et al.* (2001b).

Hatching in the incubator tank occurred in the morning between 08:00 and 12:00 for 94% of the crabs. Incubation time at 27°C corresponded with that observed by Hai *et al.* (2001) (9-12 days at 25-31°C) and for the majority of batches did not vary by more than 24 hours (Table 1). Moreover, the small variance in the increase in egg size from extrusion to hatching between different crabs (Table 1) means that, along with ontogeny of the embryo, time after extrusion and egg diameter could be used as a tool to predict the hatch date. This has important implications for the preparation of the hatchery and enrichment of live food.

## Conclusion

The relatively high fecundity and limited seasonality of spawning of South African *Scylla serrata* may be an adaptation to the variable nature of South African estuaries, of which approximately 70% are closed by barrier sand bars that open intermittently (Bell *et al.* 2001; Viljoen and Cyrus 2002). The spawning characteristics of South African *Scylla serrata* are nevertheless comparable to those observed for the genus throughout its distribution. This should be considered when formulating future fisheries management or conservation policy if these are to be adapted from those established elsewhere.

Using the fecundity data, the average percent hatching success and the percent females that spawn on a monthly basis, a total of 1 374 488 first zoea (Z1) larvae could be expected per kg of female per month. For practical aquaculture purposes, maintaining a relatively small number of female broodstock (approximately 20 animals in this study) under the correct artificial conditions could ensure a regular, year-round supply of eggs for supplying a hatchery. The comparatively high fecundity of the crabs means that relatively few individuals need to be caught from the wild for stocking commercial hatcheries - as opposed to penaeid shrimp for example (Wickins and Lee 2002). The crabs spawned easily with little requirement for special diets or environmental manipulation. The life cycle has also been closed in captivity (Quinitio *et al.* 2001). This implies that domestication of the broodstock will not be difficult, which could reduce the dependence on wild caught broodstock in the future.

## Acknowledgements

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# Chapter 5

## **Optimal first feed organism for South African mud crab *Scylla serrata* (Forskål) larvae**

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| <b>Manuscript submitted to Aquaculture International</b> |
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## Abstract

It is not known whether rotifers or *Artemia* nauplii are the best first food for South African mud crab *Scylla serrata* larvae. In order to test this, larvae were fed five different test diets. These were: rotifers for the first 8 days and newly hatched EG® type *Artemia* nauplii (San Francisco Bay) from day 6 onwards (treatment R6A); newly hatched EG® type *Artemia* nauplii throughout the rearing period (treatment EG); newly hatched Vinh Chau strain (Vietnam) *Artemia* nauplii throughout the rearing period (treatment VC); decapsulated cysts of EG® type *Artemia* (treatment DECAP); or decapsulated cysts supplemented with low densities of *Artemia* EG® type *Artemia* nauplii (treatment MIX). Two experiments were conducted approximately one month apart using larvae from two different female crabs. Although results showed it is possible to rear *S. serrata* larvae through metamorphosis on *Artemia* nauplii exclusively, larval performance (development, survival and successful metamorphosis) was enhanced by the inclusion of rotifers as a first feed.

No significant difference in performance was recorded between larvae fed on the two strains of *Artemia* nauplii. Larvae fed on decapsulated cysts in treatments DECAP and MIX performed poorly, but there were indications that decapsulated cysts and other inert diets may have potential as supplements to live food in the rearing of *Scylla serrata* larvae.

## Introduction

The estuarine mud crab *Scylla serrata* grows rapidly to a large size, accepts a wide range of diets during the post larval stages and commands a high price on the international market (Agbayani 2001; Williams and Primavera 2001). In South Africa, indigenous mud crab populations cannot sustain commercial harvesting for aquaculture (Robertson 1996) and for this reason *Scylla serrata* has been identified as a potential aquaculture species. Existing mud crab aquaculture is limited by a dependence on wild-caught juveniles (Le Vay 2001; Wickins and Lee 2002). Technology for mass rearing of the larvae must be established as a prerequisite for farming. Although some progress has been made in larval rearing technology worldwide, results are still highly variable and generally disappointing. A contributing factor is that little is known about the feeding and nutritional requirements of the larvae. Only one (unpublished) report describes the feeding of *Scylla serrata* larvae in South Africa (Du Plessis 1971).

Rotifers (usually *Brachionus* spp.) are used as a first feed in the diet of mud crab larvae (Takeuchi *et al.* 2000; Djunaidah *et al.* 2001a; Mann *et al.* 2001; Qunitio *et al.* 2001;

Hamasaki *et al.* 2002a, b; Suprayudi *et al.* 2002a). They are small, are easily captured and digested by larvae and can be enriched with essential nutrients (Watanabe *et al.* 1978; Dhert 1996). The use of rotifers as a reference diet for research is, however, undesirable as the nutritional and microbial qualities of rotifers are subject to substantial fluctuations (Skjermo and Vadstein 1993; Dhert 1996). In addition, despite recent advances, rotifer culture is difficult, expensive, labour intensive and cultures are vulnerable to periodic and unpredictable crashes (Fulks and Main 1991; Fu *et al.* 1997; Suantika 2001).

Copepods have been used as an alternative to rotifers with some success, but are difficult to culture reliably (Toledo *et al.* 1998). *Scylla* larvae in other countries have been successfully reared on diets of *Artemia* nauplii alone (Ong 1966; Brick 1974; Heasman and Fielder 1983; Fielder *et al.* 1988; Mann and Parlato 1995; Baylon and Failaman 1997; Williams *et al.* 1998; Zeng 1998; Nghia *et al.* 2001b). Du Plessis (1971) reared South African *Scylla serrata* on *Artemia* nauplii and preliminary experiments (unpublished work by the authors) indicated that larvae could be successfully reared (30-50% survival to megalopa) using this diet. Survival of *Scylla* and other crab larvae can be enhanced by choosing the correct strain of *Artemia* (Johns *et al.* 1980; Mann *et al.* 2001). Success has recently been achieved in larval rearing trials of *S. paramamosain* using the Vinh Chau (VC) strain of *Artemia franciscana* in Vietnam (Nghia *et al.* 2001b). Mud crabs of all zoal instars have been observed to actively pursue and capture unhatched *Artemia* cysts (Truong Trong Nghia, College of Aquaculture and Fisheries, Can Tho University, Can Tho City, Vietnam pers. comm.; personal observation by the senior author). The larvae then attempt to consume the cyst but are unable to penetrate the chitin impregnated alveolar layer of the capsule. This layer can be completely dissolved by oxidation treatment with hypochlorite (Van Stappen 1996). The resulting decapsulated cysts have a diameter of 200-250  $\mu\text{m}$ , half the size of newly hatched *Artemia* nauplii (470-550  $\mu\text{m}$ ). Decapsulated cysts have a similar biochemical composition to live nauplii in all the major nutrients, have more energy than newly hatched *Artemia*, are highly digestible and contain high quality protein (Vanhaecke *et al.* 1983; Léger *et al.* 1987; Pector *et al.* 1994; Garcia-Ortega *et al.* 1998 2001). They have been used successfully as a larval diet for several crustacean species, can be used as a replacement for *Artemia* nauplii in ornamental fish culture and are used as a reference diet for the rearing of African catfish (*Clarias gariepinus*) larvae (Pector *et al.* 1994; Stael *et al.* 1995; Ribeiro and Jones 1998; Lim *et al.* 2002).

The aim of this study was to determine whether *Artemia* nauplii or decapsulated *Artemia* cysts could replace rotifers as a first food for *S. serrata* larvae. If so, this would

simplify experimental procedures and have positive implications for the development of mass rearing technology.

## Materials and methods

### *Systems and procedures*

The study was carried out at Mtunzini Prawn Farm on the subtropical east coast of South Africa. The experiments were undertaken in an insulated, air conditioned laboratory. Identical 2 L cylindro-conical plastic jars were used as rearing containers. Filtered (1µm) seawater (32 g/L) was used in all trials and was replaced daily. Temperature was maintained at 29-30°C by standing the jars in a heated water bath. The seawater was treated daily with 5 mg/L oxytetracycline-hydrochloride as a prophylactic measure against microbial infections. The water was aerated using open-ended plastic air tubes. Photoperiod was maintained at 16L: 8D with white fluorescent tubes.

Broodstock females were caught in the Umlalazi estuary and matured in a black, bare bottomed, 5x4x1 m deep concrete tank at a density of 0.5 crabs/m<sup>2</sup>. The temperature of the seawater fluctuated between 19 and 25°C and was recirculated through a biofilter. Subdued light was provided on a 14 L : 10 D photoperiod. Crabs were fed *ad libitum* on fish roe, mussel, shrimp and chopped squid on successive days. Uneaten food and faeces were siphoned from the maturation tank daily. Brood crabs extruded eggs in sand filled containers that were placed on the bottom of the maturation tank. Once a brood crab was observed to be carrying eggs it was placed in a 60 L glass aquarium for observation and prophylactic treatment of the eggs. The berried crab was then transferred to a black, 600 L covered fibreglass tank provided with recirculated, UV sterilised seawater at 27°C. Larvae generally hatched 288 hours after spawning. Detailed information on broodstock maintenance and spawning is provided in Davis *et al.* (2003b).

One hour after the beginning of the hatching process, positively photo-tactic larvae were individually selected with a wide bore pipette and stocked to a density of 67/L (120 larvae per bottle) in the rearing containers. Each day, the contents of the rearing containers was gently poured into a 3 L bowl and the surviving larvae counted while being pipetted into new containers containing fresh seawater. The “new” containers were incubated beforehand in the same water bath as the “old” rearing vessels to equilibrate temperature. Ammonia (NH<sub>4</sub><sup>+</sup>-N)

was maintained at 0-0.05 mg/L; nitrite (NO<sub>2</sub>-N) at 0-0.05 mg/L; pH at 8.4-8.7; salinity at 32-34 mg/L and oxygen at 8.0-8.2 mg/L.

Development was monitored every three days by identifying the zoeal instar stage of each larva and assigning it a value: first zoea (Z1) =1; second zoea (Z2) =2, etc. to megalopa (M) = 6. To compare development in each treatment an average larval stage index (LSI) (n = 9) was calculated as described by Millamena and Bangcaya (2001).

Zoea 5 larvae first moulted (metamorphosed) into megalopae at 15 days after hatch (DAH) and were removed from the system to prevent cannibalism on the remaining Z5 larvae. Survival of the zoeae is thus presented up to 15 DAH. The total number of megalopae produced within each treatment group was calculated at 18 DAH. Survival of the larvae to the megalopa stage was calculated in two ways: firstly as the number of Z1 surviving the moult to megalopa (M/Z1) and secondly as the number of Z5 surviving the moult to megalopa (M/Z5). The former calculation takes account of survival throughout the rearing process whereas the latter calculation focuses on the process of metamorphosis.

The larvae used for each experiment were obtained from two different crabs. The experiments were conducted approximately one month apart and experimental procedure was identical.

### *Food and feeding*

Rotifers (*Brachionus plicatilis*) were cultured outdoors in a 2600 L semi-continuous recirculating system. Rotifers were fed a mixture of *Chlorella* spp. and Culture Selco® (INVE Aquaculture, Belgium). They were harvested daily through a 60 µm screen, rinsed and enriched with *Tetraselmis* spp. at a density of 5x10<sup>6</sup> cells/ml for 3 hours (Dhert 1996). The water in the enrichment vessel was slowly heated to 29°C to avoid exposing the rotifers to thermal shock when they were added to the culture vessels. The enriched rotifers were rinsed and suspended in clean sea water at 29°C before being fed to the larvae at a density of 40 rotifers/ml. *Artemia* nauplii were hatched, rinsed and stored according to Van Stappen (1996) and Merchie (1996). Two types of *Artemia* were used, EG® type (INVE Aquaculture, Belgium) *Artemia franciscana* and Vinh Chau (Vietnam) strain *Artemia franciscana*. EG® type cysts of the same batch as above were decapsulated and dehydrated in brine for storage as described by Van Stappen (1996).

Two independent feeding experiments were undertaken, one month apart, which each compared 4 treatments with 9 replicates in each. The 9 replicates were divided into 3 groups of

3 rearing vessels each. The position of these groups was randomized within the experimental grid.

The following diets were presented to the larvae at 09:00 every morning unless otherwise stated:

Rotifers and *Artemia* (R6A): Rotifers (40/ml) for the first 8 days and then EG type *Artemia* nauplii (15/ml) introduced on day 6. EG *Artemia* (EG): Newly hatched EG type *Artemia* nauplii fed at an approximate density of 15/ml throughout the rearing process. Vinh Chau *Artemia* (VC): Newly hatched VC type *Artemia* nauplii fed throughout the rearing process at the same density as treatment EG. Decapsulated cysts: To ensure that cysts were available to the larvae, decapsulated EG type cysts were rehydrated in seawater 1 hour prior to feeding and presented to the larvae every three hours at a density of 15 cysts/L. In the first experiment the cysts were presented alone (treatment DECAP). In the second experiment the cysts were fed as above (10/ml) and supplemented with EG type *Artemia* nauplii at a density of 0.5 nauplii/ml (treatment MIX).

### *Fatty acid analysis*

Larvae from three of the nine replicates of each treatment in experiment 1 were sacrificed at day 15. The larvae were then freeze dried and both were subjected to Fatty Acid Methyl Esterase (FAME) analysis at the Laboratory for Aquaculture & *Artemia* Reference Center, Ghent University, Belgium. The analysis - a modified procedure of Lepage and Roy (1984) - was conducted as described by Coutteau and Sorgeloos (1995). The live food was similarly preserved and analysed.

### *Statistical analyses*

Percent values were converted into fractions by dividing by 100. Homogeneity of variance was tested with the Levene statistic. If no significant differences were detected between the variances, the data were submitted to a one-way ANOVA. The Tukey HSD post-hoc analysis was used to detect differences between means and to indicate areas of significant difference.

If significant differences were detected between variances, the data were transformed using either logarithmic or arcsine-square root transformations. If heterogeneity was still detected, non parametric analyses were used to detect significant differences between means (Sokal and Rohlf 1995). Kruskal-Wallis H was used to detect whether there were significant

differences between the means and a multiple comparison of mean ranks for all groups was used to identify areas of significant difference (Lehmann 1998).

## Results

### *Zoeal development*

Larvae in both experiments developed most rapidly when subjected to treatment R6A and least rapidly when fed decapsulated cysts (treatment DECAP) or when these were incorporated into the diet (treatment MIX) (Table 1).

**Table 1.** Average Larval Stage Index (LSI) values  $\pm$  standard deviation for *Scylla serrata* larvae in Experiment 1 and 2. R6A = Rotifers fed for the first 8 DAH and EG type *Artemia* nauplii fed from 6 DAH; EG = EG type *Artemia* nauplii fed throughout; VC = Vin-Chau (Vietnam) type *Artemia* nauplii fed throughout; DECAP = decapsulated EG type cysts fed throughout; MIX = Decapsulated EG cysts plus low density (0.5/ml) EG nauplii fed throughout. On each day, within each experiment, LSI values that do not share the same letter are statistically significantly different ( $p \leq 0.05$ ;  $n = 9$ ).

| Experiment 1 |                              |                              |                               |                               |                               |
|--------------|------------------------------|------------------------------|-------------------------------|-------------------------------|-------------------------------|
|              | Day 3                        | Day 6                        | Day 9                         | Day 12                        | Day 15                        |
| R6A          | 1.89 $\pm$ 0.37 <sup>a</sup> | 2.11 $\pm$ 0.39 <sup>a</sup> | 3.56 $\pm$ 0.40 <sup>a</sup>  | 4.54 $\pm$ 0.52 <sup>a</sup>  | 5.16 $\pm$ 0.55 <sup>a</sup>  |
| EG           | 1.81 $\pm$ 0.38 <sup>a</sup> | 2.20 $\pm$ 0.42 <sup>a</sup> | 2.91 $\pm$ 0.32 <sup>ab</sup> | 3.49 $\pm$ 0.33 <sup>a</sup>  | 4.68 $\pm$ 0.40 <sup>a</sup>  |
| VC           | 1.80 $\pm$ 0.39 <sup>a</sup> | 2.28 $\pm$ 0.41 <sup>a</sup> | 2.92 $\pm$ 0.49 <sup>ab</sup> | 3.55 $\pm$ 0.45 <sup>a</sup>  | 4.49 $\pm$ 0.49 <sup>a</sup>  |
| DECAP        | 1.78 $\pm$ 0.37 <sup>a</sup> | 1.85 $\pm$ 0.47 <sup>a</sup> | 2.62 $\pm$ 0.45 <sup>b</sup>  | 3.22 $\pm$ 0.45 <sup>b</sup>  | 3.79 $\pm$ 0.50 <sup>b</sup>  |
| Experiment 2 |                              |                              |                               |                               |                               |
| R6A          | 1.84 $\pm$ 0.04 <sup>a</sup> | 2.75 $\pm$ 0.15 <sup>a</sup> | 3.64 $\pm$ 0.25 <sup>a</sup>  | 4.77 $\pm$ 0.35 <sup>a</sup>  | 5.16 $\pm$ 0.40 <sup>a</sup>  |
| EG           | 1.62 $\pm$ 0.44 <sup>a</sup> | 1.86 $\pm$ 0.23 <sup>b</sup> | 2.53 $\pm$ 0.24 <sup>ab</sup> | 3.81 $\pm$ 0.20 <sup>ab</sup> | 4.76 $\pm$ 0.22 <sup>ab</sup> |
| VC           | 1.76 $\pm$ 0.15 <sup>a</sup> | 2.06 $\pm$ 0.17 <sup>b</sup> | 3.16 $\pm$ 0.45 <sup>ab</sup> | 3.84 $\pm$ 0.76 <sup>ab</sup> | 4.50 $\pm$ 0.58 <sup>ab</sup> |
| MIX          | 1.43 $\pm$ 0.17 <sup>a</sup> | 1.93 $\pm$ 0.27 <sup>b</sup> | 2.46 $\pm$ 0.27 <sup>b</sup>  | 3.68 $\pm$ 0.29 <sup>b</sup>  | 4.40 $\pm$ 0.35 <sup>b</sup>  |

In experiment 1, no significant differences in the rate of development (indicated by the LSI value) were recorded between any of the treatments at 3 and 6 DAH. At 9 DAH a significantly lower LSI value was recorded for larvae in treatment DECAP than for larvae in treatment R6A ( $p < 0.05$ ). At 12 and 15 DAH, the LSI value for larvae in treatment DECAP was significantly lower than for larvae in all other treatments ( $p \leq 0.02$ ).

In experiment 2, no significant differences in LSI value were recorded for larvae in any of the treatments at 3 DAH. At 6 DAH, a significantly higher LSI value was recorded for larvae in treatment R6A than for larvae in the other treatments. The LSI value for larvae in treatment MIX was significantly lower than for larvae in treatment R6A until DAH 15 ( $p \leq 0.02$ ). There was no significant difference in LSI between larvae in treatment R6A and treatments EG and VC from 9 to 15 DAH.

### *Zoeal survival*

In experiment 1, no significant differences in larval survival between treatments were detected until 7 DAH (Table 2). High mortality was recorded for the larvae in treatment DECAP from 4 DAH and percent survival was consequently significantly lower than that of larvae in treatment R6A from 8 DAH until 15 DAH ( $p \leq 0.02$ ) and that of larvae in treatment EG ( $p \leq 0.04$ ) at 15 DAH. Percent survival of the larvae in treatment R6A was highest throughout the experiment; more than double that of larvae in treatment VC by day 15. No significant differences in survival of the zoeae were, however, detected between larvae in treatment R6A and the treatments receiving *Artemia* nauplii throughout. Similarly, although survival of the larvae was low in the DECAP treatment, the level was not significantly lower than larvae in the EG and VC treatments.

Overall survival in experiment 3 was lower than experiment 1 (Table 2). No significant differences in larval survival were recorded between any of the treatments up to 5 DAH. Relatively high mortality was recorded for larvae in treatment MIX from DAH 5. From DAH 6 to 10, survival of the larvae in treatment MIX was significantly lower than in treatment R6A ( $p \leq 0.03$ ) (Table 1). As in experiment 1, the highest survival rate was recorded for larvae in the R6A treatment.

**Table 2.** Average percent survival of *Scylla serrata* zoeae  $\pm$  standard deviation in Experiment 1 and 2. R6A = Rotifers fed for the first 8 DAH and EG type *Artemia* nauplii fed from 6 DAH; EG = EG type *Artemia* nauplii fed throughout; VC = Vin-Chau (Vietnam) type *Artemia* nauplii fed throughout; DECAP = decapsulated EG type cysts fed throughout; MIX = Decapsulated EG cysts plus low density EG nauplii fed throughout. On each day, within each experiment, survival values that do not share the same letter in superscript are statistically significantly different ( $p < 0.05$ ;  $n = 9$ ).

| Experiment 1 |                          |                           |                           |                           |                           |                           |                           |                           |                           |                           |                           |
|--------------|--------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
|              | Day3                     | Day6                      | Day7                      | Day8                      | Day9                      | Day10                     | Day11                     | Day12                     | Day13                     | Day14                     | Day15                     |
| R6A          | 92 $\pm$ 14 <sup>a</sup> | 79 $\pm$ 16 <sup>a</sup>  | 77 $\pm$ 16 <sup>a</sup>  | 74 $\pm$ 16 <sup>a</sup>  | 71 $\pm$ 15 <sup>a</sup>  | 69 $\pm$ 15 <sup>a</sup>  | 66 $\pm$ 16 <sup>a</sup>  | 65 $\pm$ 16 <sup>a</sup>  | 61 $\pm$ 15 <sup>a</sup>  | 59 $\pm$ 17 <sup>a</sup>  | 58 $\pm$ 18 <sup>a</sup>  |
| EG           | 91 $\pm$ 8 <sup>a</sup>  | 80 $\pm$ 10 <sup>a</sup>  | 66 $\pm$ 26 <sup>a</sup>  | 61 $\pm$ 26 <sup>ab</sup> | 58 $\pm$ 25 <sup>ab</sup> | 56 $\pm$ 25 <sup>ab</sup> | 52 $\pm$ 25 <sup>ab</sup> | 51 $\pm$ 25 <sup>ab</sup> | 50 $\pm$ 24 <sup>ab</sup> | 49 $\pm$ 24 <sup>ab</sup> | 49 $\pm$ 24 <sup>ab</sup> |
| VC           | 91 $\pm$ 10 <sup>a</sup> | 79 $\pm$ 16 <sup>a</sup>  | 72 $\pm$ 19 <sup>a</sup>  | 66 $\pm$ 19 <sup>ab</sup> | 58 $\pm$ 19 <sup>ab</sup> | 49 $\pm$ 19 <sup>ab</sup> | 44 $\pm$ 19 <sup>ab</sup> | 40 $\pm$ 20 <sup>ab</sup> | 36 $\pm$ 20 <sup>ab</sup> | 30 $\pm$ 21 <sup>ab</sup> | 26 $\pm$ 22 <sup>ab</sup> |
| DECAP        | 88 $\pm$ 9 <sup>a</sup>  | 55 $\pm$ 13 <sup>a</sup>  | 44 $\pm$ 15 <sup>a</sup>  | 32 $\pm$ 17 <sup>b</sup>  | 28 $\pm$ 17 <sup>b</sup>  | 24 $\pm$ 16 <sup>b</sup>  | 22 $\pm$ 15 <sup>b</sup>  | 16 $\pm$ 13 <sup>b</sup>  | 11 $\pm$ 12 <sup>b</sup>  | 8 $\pm$ 12 <sup>b</sup>   | 6 $\pm$ 8 <sup>b</sup>    |
| Experiment 2 |                          |                           |                           |                           |                           |                           |                           |                           |                           |                           |                           |
| R6A          | 92 $\pm$ 4 <sup>a</sup>  | 88 $\pm$ 6 <sup>a</sup>   | 83 $\pm$ 9 <sup>a</sup>   | 74 $\pm$ 13 <sup>a</sup>  | 68 $\pm$ 14 <sup>a</sup>  | 65 $\pm$ 14 <sup>a</sup>  | 61 $\pm$ 14 <sup>a</sup>  | 57 $\pm$ 14 <sup>a</sup>  | 47 $\pm$ 20 <sup>a</sup>  | 38 $\pm$ 13 <sup>a</sup>  | 34 $\pm$ 14 <sup>a</sup>  |
| EG           | 93 $\pm$ 4 <sup>a</sup>  | 71 $\pm$ 7 <sup>ab</sup>  | 61 $\pm$ 11 <sup>ab</sup> | 55 $\pm$ 13 <sup>ab</sup> | 49 $\pm$ 14 <sup>ab</sup> | 45 $\pm$ 16 <sup>ab</sup> | 41 $\pm$ 16 <sup>a</sup>  | 31 $\pm$ 18 <sup>a</sup>  | 28 $\pm$ 18 <sup>a</sup>  | 24 $\pm$ 15 <sup>a</sup>  | 20 $\pm$ 15 <sup>a</sup>  |
| VC           | 87 $\pm$ 25 <sup>a</sup> | 71 $\pm$ 17 <sup>ab</sup> | 58 $\pm$ 20 <sup>ab</sup> | 50 $\pm$ 21 <sup>ab</sup> | 45 $\pm$ 23 <sup>ab</sup> | 38 $\pm$ 22 <sup>ab</sup> | 34 $\pm$ 22 <sup>a</sup>  | 29 $\pm$ 20 <sup>a</sup>  | 24 $\pm$ 15 <sup>a</sup>  | 20 $\pm$ 18 <sup>a</sup>  | 16 $\pm$ 16 <sup>a</sup>  |
| MIX          | 87 $\pm$ 13 <sup>a</sup> | 68 $\pm$ 19 <sup>b</sup>  | 58 $\pm$ 19 <sup>b</sup>  | 42 $\pm$ 20 <sup>b</sup>  | 36 $\pm$ 21 <sup>b</sup>  | 32 $\pm$ 21 <sup>b</sup>  | 28 $\pm$ 21 <sup>a</sup>  | 26 $\pm$ 20 <sup>a</sup>  | 23 $\pm$ 19 <sup>a</sup>  | 16 $\pm$ 16 <sup>a</sup>  | 13 $\pm$ 23 <sup>a</sup>  |

*Survival through metamorphosis*

In experiment 1 no megalopae were produced in the DECAP treatment (Table 3). A significantly higher proportion of Z1 larvae survived metamorphosis to megalopa in treatment R6A than in treatment VC ( $p = 0.03$ ). Although percent metamorphosis of Z5 larvae to megalopa was higher in treatment R6A than the other treatments, the difference was not significant. There was no significant difference in survival through metamorphosis for larvae in treatments EG and VC.

In experiment 2, only ten megalopae were produced in the MIX treatment. This was significantly less than the number produced in the other treatments ( $p = 0.001$ ). A significantly higher proportion of Z1 and Z5 survived metamorphosis to megalopa in treatment R6A than in the other treatments ( $p = 0.02$ ).

**Table 3.** Average percent survival through metamorphosis of *Scylla serrata* larvae  $\pm$  standard deviation in Experiments 1 and 2. M/Z1= % metamorphosis of zoea 1 to megalopa; M/Z5 = % metamorphosis of zoea 5 to megalopa. R6A = Rotifers fed for the first 8 DAH and EG type *Artemia* nauplii fed from 6 DAH; EG = EG type *Artemia* nauplii fed throughout; VC = Vin-Chau (Vietnam) type *Artemia* nauplii fed throughout; DECAP = decapsulated EG type cysts fed throughout; MIX = Decapsulated EG cysts plus low density EG nauplii fed throughout. On each day, within each experiment, survival values that do not share the same letter are statistically significantly different ( $p \leq 0.05$ );  $n = 6$  (experiment 1);  $n = 9$  (experiment 2).

| Experiment 1 | Percent survival M/Z1     | Percent survival M/Z5    | Experiment 2 | Percent survival M/Z1   | Percent survival M/Z5    |
|--------------|---------------------------|--------------------------|--------------|-------------------------|--------------------------|
| R6A          | 28 $\pm$ 19 <sup>a</sup>  | 56 $\pm$ 22 <sup>a</sup> | R6A          | 15 $\pm$ 6 <sup>a</sup> | 37 $\pm$ 22 <sup>a</sup> |
| EG           | 15 $\pm$ 12 <sup>ab</sup> | 23 $\pm$ 23 <sup>a</sup> | AC           | 3 $\pm$ 3 <sup>b</sup>  | 11 $\pm$ 11 <sup>b</sup> |
| VC           | 7 $\pm$ 7 <sup>b</sup>    | 26 $\pm$ 31 <sup>a</sup> | VC           | 2 $\pm$ 2 <sup>b</sup>  | 8 $\pm$ 5 <sup>b</sup>   |
| DECAP        | 0 $\pm$ 0 <sup>c</sup>    | 0 $\pm$ 0 <sup>b</sup>   | MIX          | 0 $\pm$ 1 <sup>c</sup>  | 4 $\pm$ 1 <sup>c</sup>   |

*FAME analysis*

Rotifers contained lower gross FAME, but higher DHA and DHA/EPA values than the *Artemia* (Table 4). The VC *Artemia* contained higher FAME, EPA and sum (n-3) than the EG *Artemia* but a lower ARA/EPA ratio. In contrast, there was little difference in EFA levels between larvae in the different treatments.

**Table 4.** Fatty acid profile (mg/g) of live food and *Scylla serrata* larvae at 15 DAH in Experiment 1. EPA = Eicosapentaenoic acid (20:5(n-3)); DHA = Docosahexaenoic acid (22:(6n-3)); ARA = Arachidonic acid (20:4(n-6)). R6A = Rotifers fed for the first 8 DAH and EG type *Artemia* nauplii fed from 6 DAH; EG = EG type *Artemia* nauplii fed throughout; VC = Newly hatched Vin-Chau (Vietnam) type *Artemia* nauplii fed throughout; \*  $\geq 20:3(n-3)$ ; \*\*  $\geq 18:2(n-6)$ .

| Highly Unsaturated Fatty Acid levels (mg/g) |            |      |     |     |                     |                      |         |         |
|---------------------------------------------|------------|------|-----|-----|---------------------|----------------------|---------|---------|
|                                             | Gross FAME | EPA  | DHA | ARA | $\Sigma(n-3)$ HUFA* | $\Sigma(n-6)$ HUFA** | DHA/EPA | ARA/EPA |
| Live Food                                   |            |      |     |     |                     |                      |         |         |
| Rotifers                                    | 49.5       | 6.9  | 5.3 | 1.0 | 11.0                | 7.6                  | 0.8     | 0.1     |
| EG Nauplii                                  | 193.3      | 6.5  | 0.6 | 2.0 | 8.1                 | 14.9                 | 0.03    | 0.3     |
| VC Nauplii                                  | 149.2      | 19.2 | 0.6 | 6.9 | 20.5                | 15.1                 | 0.03    | 0.4     |
| Larvae                                      |            |      |     |     |                     |                      |         |         |
| R6A                                         | 96.1       | 11.5 | 3.8 | 1.0 | 17.5                | 8.8                  | 0.3     | 0.09    |
| EG                                          | 102.3      | 9.4  | 2.9 | 0.4 | 14                  | 8.3                  | 0.3     | 0.04    |
| VC                                          | 90.2       | 7.4  | 1.4 | 0.3 | 9.7                 | 6.9                  | 0.2     | 0.04    |

## Discussion

### *Artemia* nauplii as an alternative to rotifers

The absence of rotifers in the diet was associated with slower development, higher zoal mortality and lower survival through metamorphosis to megalopa (Tables 1, 2 and 3). Rotifers (0.5  $\mu\text{g}$  and 45-200  $\mu\text{m}$ ) are significantly smaller than *Artemia* (2.7  $\mu\text{g}$  and 428-517  $\mu\text{m}$ ) and are less vigorous swimmers (Léger *et al.* 1986; Harvey and Epifanio 1997). Measurements of the feeding appendages of *Scylla serrata* larvae by Chang and Wu (1985) suggest that the optimum food size for Z1 larvae ranges from 100-200  $\mu\text{m}$ . The ability of the Z1 larvae to efficiently consume *Artemia* could thus have been limited by the functional morphology of their feeding appendages. Early crab larvae (Z1-Z2) show a clear preference for (slower moving) rotifers and there is evidence that although the larvae of many species accept *Artemia* nauplii as a first food, they often face problems ingesting and handling them (Léger *et al.* 1987; Harvey and Epifanio 1997). Despite being smaller than the EG nauplii ( $\pm 486 \mu\text{m}$ ), the VC nauplii ( $\pm 457 \mu\text{m}$ ) did not confer an advantage to the larvae.

Rotifers are also more digestible than *Artemia* (Watanabe *et al.* 1978) and early crustacean larvae exhibit low levels of enzyme activity and digestive capacity (Ribeiro and

Jones 2000; Le Vay *et al.* 2001a). Features such as the gastric mill, highly vacuolated epithelial cells of the midgut and a well developed hepatopancreas start to appear only at the Z3 stage in *Scylla serrata* (Li 1999). Many partially consumed *Artemia* were observed in the EG and VC treatments during the early larval stages. Usually only the head and appendages were eaten, leaving the enzyme containing gut intact. This confirms the observations of Zeng and Li (1999).

Although the crab larvae that were only provided with *Artemia* generally performed poorer than those receiving rotifers, the differences were not always significant. This indicates that the Z1 larvae were indeed capable of consuming *Artemia* nauplii and sustaining growth and survival to the megalopa stage without rotifers albeit with lower efficiency. Crain (1999) suggested that although the size of the mouthparts of small crab zoeae may appear limiting, the maxillules are capable of remarkable plasticity of movement, enabling the consumption of large, struggling prey such as *Artemia*. These results are promising in terms of the potential for introducing *Artemia* into the diet as a supplement to rotifers at an early stage of development.

### *Decapsulated cysts as an alternative to rotifers*

Feeding dehydrated, decapsulated *Artemia* cysts to the larvae resulted in poor performance despite supplementation with *Artemia* nauplii (Tables 1, 2 and 3). Significantly slower development was recorded and none of the larvae in the DECAP treatment survived metamorphosis to megalopa. Unlike intact cysts, decapsulated cysts rapidly settle out of the water column despite reasonably high (16 ml air/min) aeration. Early mud crab larvae are inefficient predators (Heasman and Fielder 1983; Zeng 1998; Zeng and Li 1999). Even though the inert nature of the *Artemia* cysts should have made them easier to catch and consume than nauplii, a low density in the water column would have reduced the encounter rate between the larvae and the cysts.

A possible solution could be to add the cysts at more regular intervals, but there is a limit to the amount of cysts that can be added to the rearing vessels without compromising water quality. In larger (100 L cylindroconical) vessels, higher aeration rates were used, resulting in improved cyst availability. This did not improve larval survival significantly (unpublished data). Animals which perform well on decapsulated cysts such as shrimp post-larvae and the larvae of ornamental fish and African catfish are much more efficient feeders than mud crab zoeae, able to not only find and consume their ration soon after feeding, but are

also able to feed off the bottom of the culture vessel (Sumitra-Vijayaraghavan *et al.* 1988; Stael *et al.* 1995; Ribeiro and Jones 1998).

Despite the poor performance of the larvae receiving decapsulated cysts, the observation that not all the larvae died in the DECAP treatment has important implications for the future use of artificial diets in mud crab larval nutrition. Supplementation of the cysts with low densities of *Artemia* nauplii in the MIX treatment also improved performance (Tables 1 and 2), indicating that decapsulated cysts may be useful in the diet as a supplement to live feed.

#### Artemia source and EFA content

The EG type *Artemia* were slightly more effective than the VC strain although differences in performance were not significant. The use of different geographic strains of *Artemia* has been shown to have a marked effect on the survival and development of crab larvae, including *Scylla serrata* (Johns *et al.* 1980; Seidel *et al.* 1982; Naihong *et al.* 1999; Mann *et al.* 2001). The main reasons for this have been attributed to differences in nutritional content, particularly of the essential fatty acids (EFA). Enrichment of *Artemia* strains poor in eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3) have been shown to improve survival, growth and stress resistance in crab and other crustacean larvae, including *Scylla* (Levine and Sulkin 1984; Léger *et al.* 1986; Naihong *et al.* 1999, Sulkin and McKeen 1999; Kobayashi *et al.* 2000; Takeuchi *et al.* 2000; Suprayudi *et al.* 2002b). High levels of EPA, total (n-3) and total (n-6) HUFA contained in the VC cysts (Table 5) did not, however, result in enhanced larval performance suggesting that the correct amounts and balances of dietary EFAs may hold little benefit to mud crab larvae, if the vector for those fatty acids is an unsuitable feed organism.

It is, however, not only the gross content of EFA that has an effect on crustacean larvae (González-Felix *et al.* 2002), but also the balance of those fatty acids to one another (Rees *et al.* 1994). The low DHA/EPA and arachidonic acid (20:4(n-6)) (ARA)/EPA ratio of the VC *Artemia* was reflected by a correspondingly low ratio in the larvae and may have compromised growth and survival. High levels of either one of EPA or DHA in the diet or an incorrect ARA/EPA balance has a negative effect on crustacean larvae (González-Baro and Pollero 1998; Glencross and Smith 2001a).

## **Conclusion**

The results of these experiments imply that rotifers cannot be completely replaced as a feed during the early life stages of *S. serrata* larvae. The fact that the larvae do, however, accept *Artemia* nauplii and decapsulated cysts as early as the zoea 1 stage suggests that larval performance could be enhanced by supplementing rotifers with these feeds early in the rearing process.

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# **Chapter 6**

## **Optimum time for weaning South African *Scylla serrata* (Forskål) larvae from rotifers to *Artemia***

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### Abstract

To determine the optimum time at which to wean *S. serrata* larvae from rotifers onto *Artemia* two experiments were conducted, approximately one month apart, using larvae from two different female crabs. In the first experiment, the larvae in three treatment groups with 9 replicates each were fed rotifers for the first eight days after hatching. *Artemia* were introduced on days after hatch (DAH) 0 - during the first zoeal instar (treatment R+A); on DAH 4 – during the second zoeal instar (treatment R4A); on DAH 8 – during the third zoeal instar (treatment R8A). In a control (ROT) larvae were fed rotifers exclusively for 18 days until the completion of metamorphosis to megalopa. In the second experiment, the same four feeding schedules as in experiment 1 were used with an additional group of larvae (treatment AC) that were fed only on *Artemia* throughout the rearing period.

Similar results were recorded in the two experiments. Larvae in treatments R+A and R4A performed significantly better than those in treatments R8A, ROT and AC. This was particularly evident when examining the proportion of zoeae which successfully completed metamorphosis to megalopa. Poor performance of larvae in treatments AC and ROT implied that rotifers are needed as a first food, but that rotifers alone do not fill the nutritional requirements of *S. serrata* larvae. Poor performance of larvae in treatment R8A suggested that the diet should be supplemented with *Artemia* before the end of the zoea 3 stage.

Although results indicate that *S. serrata* larvae benefit from *Artemia* supplementation from hatch, no significant differences in performance were found between larvae in the R+A and R4A treatments. When considering the practical zootechnics of larger scale systems, it is suggested that *Artemia* be offered to South African mud crab larvae after moulting to the zoea 2 stage.

### Introduction

*Scylla* species are large, commercially important, primarily carnivorous crabs with an Indo-Pacific distribution. Mud crab farming occurs mainly in Asia, where juveniles and adults are caught from the wild and reared or fattened for the market (Yalin and Qingsheng 1994; Kathirvel 1995; Marichamy and Rajapackiam 2001; Wickins and Lee 2002). It is doubtful that this practice is sustainable (Le Vay 2001). In South Africa the natural populations are too small to support intensive and sustained fishing pressure (Robertson and Kruger 1994; Robertson 1996) and for this reason *S. serrata* has been identified as a candidate species for commercial

farming. It is recognised that the establishment of larval rearing protocols is a prerequisite for the development of the industry internationally. Despite a long history, starting with Ong (1964; 1966), research into mud crab larviculture has been sporadic and only recently has there been a concerted international effort (Wickins and Lee 2002). However, the mass rearing of larvae has not yet been developed to the extent that it is commercially viable and little has been published on the subject in the primary literature. Moreover, there is some doubt regarding the identity of the species for which information has been reported prior to the revision of the genus by Keenan *et al.* (1998).

It is known that mud crab larvae accept both rotifers and *Artemia* as prey items. Growth and survival are, however, compromised when either of the two feeds is provided alone (Baylon and Failaman 1997; Zeng and Li 1999; Takeuchi *et al.* 2000). It has therefore become common practice to start feeding the larvae on rotifers and then to switch to *Artemia* at some stage of development (Djunaidah *et al.* 2001a; Mann *et al.* 2001; Quinitio *et al.* 2001).

Suprayudi *et al.* (2002a) established that providing *Artemia* at the zoea 3 stage improved the survival and growth of Japanese *Scylla serrata*. It is not known, however, whether there are differences between the feeding requirements of *Scylla serrata* larvae from different geographical areas. The present study was conducted to establish the optimum stage at which to wean southern African *Scylla serrata* from rotifers onto *Artemia*. Such information would make an important contribution to the development of commercial aquaculture of the species throughout the Western Indian Ocean region.

## Materials and methods

### *Systems and procedures*

The study was carried out at Mtunzini Prawn Farm on the subtropical east coast of South Africa. The experiments were undertaken in an insulated, air conditioned laboratory. Identical 2 L cylindro-conical plastic jars were used as rearing containers. Filtered (1 µm) seawater (32 g/L) was used in all trials and was replaced daily. Temperature was maintained at 29-30°C by standing the jars in a heated water bath. The seawater was treated with 5 mg/L oxytetracycline-hydrochloride daily to prevent microbial infections. The water was aerated using open ended plastic air tubes. Photoperiod was maintained at 16L: 8D with white fluorescent tubes.

Broodstock females were caught in the Umlalazi estuary and matured in a black, bare bottomed, 5x4x1 m deep concrete tank at a density of 0.5 crabs/m<sup>2</sup>. The temperature of the seawater (32 g/L) fluctuated between 19 and 25°C and was recirculated through a biofilter. Subdued light was provided on a 14L: 10D photoperiod. Crabs were fed *ad libitum* on fish roe, mussel, shrimp and chopped squid on successive days. Uneaten food and faeces was siphoned from the maturation tank daily. Female brood crabs extruded eggs in sand filled containers that were placed on the bottom of the maturation tank. Berried brood crabs were caught and transferred to a 60 L glass aquarium for observation and prophylactic treatment of the eggs, where after, they were transferred into black, 600 L, covered fibreglass tanks provided with recirculated, UV sterilised seawater at 27°C. Larvae generally hatched 288 hours after spawning. Detailed information on broodstock maintenance and spawning is provided in Davis *et al.* (2003a).

One hour after hatching, positively photo-tactic larvae were individually selected with a wide bore pipette and stocked at a density of 67/L in the rearing containers (120 larvae per vessel). Each day, the contents of the rearing containers was gently poured into a 3 L bowl and the surviving larvae counted while being pipetted into new containers containing fresh seawater. The “new” containers were incubated beforehand in the same water bath as the “old” rearing vessels to equilibrate temperature. Ammonia (NH<sub>4</sub><sup>+</sup>-N) was maintained at 0 - 0.05 mg/L; nitrite (NO<sub>2</sub><sup>-</sup>-N) at 0 - 0.05 mg/L; pH at 8.4 - 8.7 and oxygen at 8.0 - 8.2 mg/L.

Two experiments were conducted approximately one month apart. Each experiment used larvae from a different female. The first and second experiments consisted of 4 and 5 treatments respectively, each with 9 replicates per treatment. The 9 replicates were divided into 3 groups of 3 containers each. The position of these groups was randomised within the experimental grid.

Development was monitored every three days by identifying the zoeal instar stage of each larva and assigning it a value: first zoea (Z1) = 1; second zoea (Z2) = 2, etc. to megalopa (M) = 6. To compare development in each treatment an average larval stage index (LSI) was calculated as described by Millamena and Bangcaya (2001).

Zoea 5 larvae first moulted (metamorphosed) into megalopae 15 days after hatch (DAH) and were removed from the system to prevent cannibalism. Survival of the zoeae is thus presented until 15 DAH. The number of megalopae produced within each treatment group was calculated at 18 DAH. Survival to metamorphosis was calculated in two ways: Firstly as the percent of Z1 surviving the moult to megalopa (M/Z1x100) and secondly as the percent of Z5 surviving the moult to megalopa (M/Z5x100). The former calculation takes account of

survival throughout the rearing process, whereas the latter calculation focuses on the metamorphosis to megalopa during which mortality is generally high (Mann *et al.* 2001; Marichamy and Rajapackiam 2001; Quintio *et al.* 2001; Hamasaki *et al.* 2002b; Suprayudi *et al.* 2002a).

### *Food and feeding*

Rotifers (*Brachionus plicatilis*) were cultured outdoors in a 2600 L semi-continuous recirculating system and fed a mixture of *Chlorella* spp. and Culture Selco® (INVE Aquaculture, Belgium). They were harvested daily through a 60 µm screen, rinsed and enriched with *Tetraselmis* spp. at a density of  $5 \times 10^6$  cells/ml for 3 hours (Dhert 1996). The water in the enrichment vessel was slowly heated to 29°C to avoid exposing the rotifers to thermal shock when they were added to the larval rearing vessels. Before being fed to the larvae, enriched rotifers were rinsed and suspended in clean sea water at 29°C.

*Artemia* nauplii (EG® type, INVE Aquaculture, Belgium) were hatched as described by Van Stappen (1996). When fed to late stage zoeae (Z3 onwards) the nauplii were enriched with Super Selco® (INVE Aquaculture, Belgium). In situations where, after harvesting, they could not immediately be fed to the larvae, both enriched metanauplii and newly hatched nauplii were kept at  $\pm 5^\circ\text{C}$  to delay development and prevent loss of nutrients (Merchie 1996). Before feeding to the crab larvae, the *Artemia* were rinsed with tap water and suspended at a known density in seawater. Rotifers and *Artemia* were fed at higher than normal densities: 40/ml and 15/ml respectively (Baylon *et al.* 2001a; Quintio *et al.* 2001; Wickins and Lee 2002) to ensure that access to prey was not a limiting factor. The larvae were fed once daily at 09:00 in the morning. In the first experiment, four different feeding schedules (treatments) were followed in which rotifers were supplemented with *Artemia* at three different zoeal stages: Z1, Z2 and Z3 at 0, 4 and 8 DAH respectively. In a control group, larvae were fed enriched rotifers throughout. In the second experiment, the treatments were repeated with the addition of a feeding schedule in which the zoeae were only provided with *Artemia* for the duration of the experiment (Fig. 1).

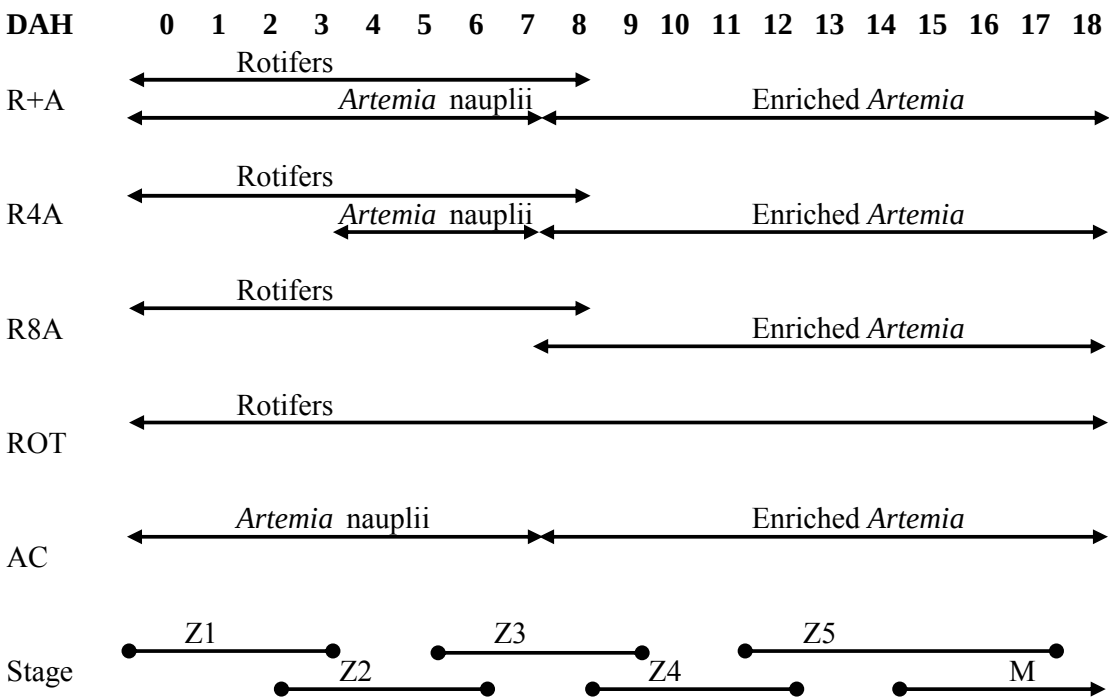


**Figure1.** Feeding schedule for *Scylla serrata* larvae. DAH = Days after hatch; Z = Zoea; M = Megalopa.

Statistical analyses

Percent values were converted into fractions by dividing by 100. Homogeneity of variance was tested with the Levene statistic. If no significant differences were detected between the variances, the data were submitted to a one-way ANOVA. The Tukey HSD post-hoc analysis was used to detect differences between the means and to indicate areas of significant difference.

If significant differences were detected between variances, the data were transformed using either logarithmic or arcsine-square root transformations. If heterogeneity was still detected, non parametric analyses were used to detect significant differences between means (Sokal and Rohlf 1995). Kruskal-Wallis H was used to detect whether there were significant differences between the means and a multiple comparison of mean ranks for all groups was used to identify areas of significant difference (Lehmann 1998).

## Results

### *Zoeal development*

Larval development followed the general pattern displayed by *Scylla* species elsewhere (Ong 1964; Heasman and Fielder 1983; Ronquillo *et al.* 1998). There were five pelagic zoeal instars, each lasting approximately three days (Fig. 1). The last zoeal instar was occasionally extended to five or six days after which the larvae metamorphosed into semi-benthic megalopae. Moulting was however not always perfectly synchronous and there was approximately one day's overlap between stages. The general pattern of larval development can be summarised as follows: first zoea (Z1) = 0 to 3 DAH; second zoea (Z2) = 3 to 6 DAH; third zoea (Z3) = 6 to 9 DAH, fourth zoea (Z4) = 9 to 12 DAH; fifth zoea (Z5) = 12-17 DAH; megalopa (M) = 15 to 21-25 DAH.

### *Rate of development*

In experiment 1, there were no significant differences in larval development rate (as indicated by the LSI value) between at 3 and 6 DAH (Table 1). However by day 9 the LSI value in the ROT treatment was significantly lower than that in the R+A and R4A treatments ( $p = 0.03$ ). This difference continued until 15 DAH ( $p < 0.05$ ). The LSI value in treatment R8A was significantly lower than that of larvae in treatments R+A and R4A on day 12 ( $p = 0.04$ ). By day 15, however, the development of larvae in treatment R8A had “caught up” to those in treatments R+A and R4A with the result that the LSI value for larvae in the ROT treatment was at this stage significantly lower than for the larvae in the other three treatments ( $p = 0.03$ ).

In experiment 2, no significant differences in larval development were recorded between any of the treatments on 3 or 6 DAH. At 9 DAH the LSI value for the larvae in the AC treatment was significantly lower than that recorded in the R+A and R4A treatments ( $p = 0.04$ ). By 12 DAH a significantly higher LSI value was recorded for larvae in treatment R4A than in treatments ROT and AC ( $p = 0.05$ ). By day 15, LSI values for larvae in both the AC and ROT treatments were significantly lower than those recorded in treatments R+A and R4A ( $p = 0.04$ ).

**Table 1.** Average Larval Stage Index (LSI) values  $\pm$  standard deviation for *Scylla serrata* larvae on significant days after hatch. R+A = *Artemia* provided on 0 DAH; R4A = *Artemia* provided on 4 DAH; R8A = *Artemia* provided on 8 DAH; ROT = Rotifers only; AC = *Artemia* only. Values in the same column (for each experiment) showing the same superscript are not significantly different. ( $p \leq 0.05$ ;  $n = 9$ ).

| Experiment 1 |                              |                              |                               |                               |                               |
|--------------|------------------------------|------------------------------|-------------------------------|-------------------------------|-------------------------------|
|              | Day 3                        | Day6                         | Day9                          | Day12                         | Day15                         |
| R+A          | 1.85 $\pm$ 0.67 <sup>a</sup> | 2.56 $\pm$ 0.14 <sup>a</sup> | 3.29 $\pm$ 0.16 <sup>a</sup>  | 4.36 $\pm$ 0.51 <sup>a</sup>  | 4.97 $\pm$ 0.55 <sup>a</sup>  |
| R4A          | 1.66 $\pm$ 0.13 <sup>a</sup> | 2.54 $\pm$ 0.21 <sup>a</sup> | 3.19 $\pm$ 0.22 <sup>a</sup>  | 4.17 $\pm$ 0.21 <sup>a</sup>  | 4.85 $\pm$ 0.28 <sup>a</sup>  |
| R8A          | 1.78 $\pm$ 0.10 <sup>a</sup> | 2.35 $\pm$ 0.18 <sup>a</sup> | 2.92 $\pm$ 0.27 <sup>ab</sup> | 3.72 $\pm$ 0.23 <sup>b</sup>  | 4.71 $\pm$ 0.20 <sup>a</sup>  |
| ROT          | 1.75 $\pm$ 0.07 <sup>a</sup> | 2.11 $\pm$ 0.13 <sup>a</sup> | 2.62 $\pm$ 0.04 <sup>b</sup>  | 3.33 $\pm$ 0.10 <sup>b</sup>  | 3.86 $\pm$ 0.19 <sup>b</sup>  |
| Experiment 2 |                              |                              |                               |                               |                               |
| R+A          | 1.83 $\pm$ 0.09 <sup>a</sup> | 2.72 $\pm$ 0.15 <sup>a</sup> | 3.68 $\pm$ 0.28 <sup>a</sup>  | 4.46 $\pm$ 0.56 <sup>ab</sup> | 5.06 $\pm$ 0.29 <sup>a</sup>  |
| R4A          | 1.87 $\pm$ 0.06 <sup>a</sup> | 2.48 $\pm$ 0.24 <sup>a</sup> | 3.65 $\pm$ 0.38 <sup>a</sup>  | 4.61 $\pm$ 0.45 <sup>a</sup>  | 5.27 $\pm$ 0.33 <sup>a</sup>  |
| R8A          | 1.88 $\pm$ 0.09 <sup>a</sup> | 2.10 $\pm$ 0.19 <sup>a</sup> | 3.00 $\pm$ 0.38 <sup>ab</sup> | 3.55 $\pm$ 0.46 <sup>ab</sup> | 4.84 $\pm$ 0.18 <sup>ab</sup> |
| ROT          | 1.64 $\pm$ 0.20 <sup>a</sup> | 2.23 $\pm$ 0.27 <sup>a</sup> | 2.91 $\pm$ 0.06 <sup>b</sup>  | 3.65 $\pm$ 0.21 <sup>b</sup>  | 4.34 $\pm$ 0.20 <sup>b</sup>  |
| AC           | 1.67 $\pm$ 0.08 <sup>a</sup> | 2.30 $\pm$ 0.36 <sup>a</sup> | 3.13 $\pm$ 0.034 <sup>b</sup> | 3.43 $\pm$ 0.56 <sup>b</sup>  | 4.10 $\pm$ 0.71 <sup>b</sup>  |

#### Daily zoeal survival

In experiment 1, there were no significant differences in larval survival between any of the four treatments up to 9 DAH (Table 2). However, from 10 DAH until the end of the experiment, high mortalities were recorded in the ROT treatment relative to the others. In contrast, survival rates of the larvae in treatments R+A and R4A remained relatively stable throughout the experiment. This resulted in significantly lower survival being recorded for larvae in the ROT treatment than for those in the R+A treatment from 10-15 DAH ( $p \leq 0.038$ ). From 12 DAH survival of larvae in both treatments R+A and R4A was significantly higher than in the ROT treatment ( $p \leq 0.03$ ). Survival of the larvae in treatment R8A generally fell between that of larvae in treatments ROT and R4A but was not significantly different to survival in any of the other treatments.

In experiment 2, high mortalities were recorded in the AC treatment between 4 and 6 DAH (Table 2). Significantly lower survival was consequently recorded for larvae in the AC treatment compared to the other treatments on 6 and 7 DAH ( $p \leq 0.04$ ). After 6 DAH mortality rates in treatments R8A and ROT increased and the mortality rate in treatment AC stabilised, reducing the differences in survival to insignificant levels. Survival of the larvae in treatment

AC remained significantly lower than in treatment R+A until 15 DAH ( $p \leq 0.04$ ). Relatively high mortality was recorded in treatments R8A, ROT and AC toward the end of the zoeal rearing period, while survival of the larvae in the R+A and R4A treatments remained relatively stable throughout. This resulted in significantly lower survival rates in the R8A, ROT and AC treatments relative to the R+A treatment on DAH 14 and 15 ( $p \leq 0.02$ ).

**Table 2.** Average percent survival  $\pm$  standard deviation of *Scylla serrata* larvae in experiments 1 and 2. R+A = *Artemia* provided on 0 DAH; R4A = *Artemia* provided at 4 DAH; R8A = *Artemia* provided at 8 DAH; ROT = Rotifers only; AC = *Artemia* only. Percent survival values for the various treatments in the same column (for each experiment) showing the same superscript are not significantly different. ( $p \leq 0.05$ ;  $n = 9$ ).

| Experiment 1 |                         |                          |                          |                           |                           |                           |                           |                           |                           |                           |                           |
|--------------|-------------------------|--------------------------|--------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
|              | Day3                    | Day6                     | Day7                     | Day8                      | Day9                      | Day10                     | Day11                     | Day12                     | Day13                     | Day14                     | Day15                     |
| R+A          | 90 $\pm$ 9 <sup>a</sup> | 83 $\pm$ 10 <sup>a</sup> | 78 $\pm$ 10 <sup>a</sup> | 73 $\pm$ 11 <sup>a</sup>  | 69 $\pm$ 12 <sup>a</sup>  | 66 $\pm$ 12 <sup>a</sup>  | 62 $\pm$ 15 <sup>a</sup>  | 59 $\pm$ 18 <sup>a</sup>  | 56 $\pm$ 17 <sup>a</sup>  | 53 $\pm$ 18 <sup>a</sup>  | 41 $\pm$ 14 <sup>a</sup>  |
| R4A          | 92 $\pm$ 3 <sup>a</sup> | 87 $\pm$ 4 <sup>a</sup>  | 82 $\pm$ 6 <sup>a</sup>  | 74 $\pm$ 6 <sup>a</sup>   | 70 $\pm$ 11 <sup>a</sup>  | 66 $\pm$ 13 <sup>ab</sup> | 61 $\pm$ 15 <sup>ab</sup> | 57 $\pm$ 17 <sup>a</sup>  | 53 $\pm$ 19 <sup>a</sup>  | 49 $\pm$ 16 <sup>a</sup>  | 37 $\pm$ 20 <sup>a</sup>  |
| R8A          | 96 $\pm$ 3 <sup>a</sup> | 89 $\pm$ 4 <sup>a</sup>  | 84 $\pm$ 6 <sup>a</sup>  | 77 $\pm$ 6 <sup>a</sup>   | 70 $\pm$ 11 <sup>a</sup>  | 59 $\pm$ 19 <sup>ab</sup> | 55 $\pm$ 17 <sup>ab</sup> | 51 $\pm$ 18 <sup>ab</sup> | 45 $\pm$ 19 <sup>ab</sup> | 41 $\pm$ 18 <sup>ab</sup> | 37 $\pm$ 19 <sup>ab</sup> |
| ROT          | 95 $\pm$ 2 <sup>a</sup> | 84 $\pm$ 3 <sup>a</sup>  | 80 $\pm$ 3 <sup>a</sup>  | 70 $\pm$ 5 <sup>a</sup>   | 62 $\pm$ 7 <sup>a</sup>   | 54 $\pm$ 10 <sup>b</sup>  | 45 $\pm$ 15 <sup>b</sup>  | 34 $\pm$ 18 <sup>b</sup>  | 29 $\pm$ 17 <sup>b</sup>  | 24 $\pm$ 15 <sup>b</sup>  | 22 $\pm$ 14 <sup>b</sup>  |
| Experiment 2 |                         |                          |                          |                           |                           |                           |                           |                           |                           |                           |                           |
| R+A          | 96 $\pm$ 2 <sup>a</sup> | 93 $\pm$ 3 <sup>a</sup>  | 89 $\pm$ 4 <sup>a</sup>  | 87 $\pm$ 5 <sup>a</sup>   | 84 $\pm$ 5 <sup>a</sup>   | 82 $\pm$ 4 <sup>a</sup>   | 81 $\pm$ 4 <sup>a</sup>   | 80 $\pm$ 5 <sup>a</sup>   | 78 $\pm$ 6 <sup>a</sup>   | 77 $\pm$ 5 <sup>a</sup>   | 74 $\pm$ 5 <sup>a</sup>   |
| R4A          | 98 $\pm$ 2 <sup>a</sup> | 90 $\pm$ 6 <sup>a</sup>  | 88 $\pm$ 6 <sup>a</sup>  | 84 $\pm$ 9 <sup>a</sup>   | 82 $\pm$ 9 <sup>a</sup>   | 80 $\pm$ 11 <sup>ab</sup> | 78 $\pm$ 11 <sup>ab</sup> | 76 $\pm$ 10 <sup>ab</sup> | 74 $\pm$ 12 <sup>ab</sup> | 72 $\pm$ 14 <sup>ab</sup> | 65 $\pm$ 13 <sup>ab</sup> |
| R8A          | 98 $\pm$ 4 <sup>a</sup> | 90 $\pm$ 8 <sup>a</sup>  | 85 $\pm$ 10 <sup>a</sup> | 82 $\pm$ 10 <sup>ab</sup> | 75 $\pm$ 14 <sup>ab</sup> | 68 $\pm$ 16 <sup>ab</sup> | 65 $\pm$ 17 <sup>ab</sup> | 61 $\pm$ 19 <sup>ab</sup> | 56 $\pm$ 20 <sup>ab</sup> | 52 $\pm$ 21 <sup>b</sup>  | 50 $\pm$ 2 <sup>b</sup>   |
| ROT          | 99 $\pm$ 2 <sup>a</sup> | 89 $\pm$ 7 <sup>a</sup>  | 89 $\pm$ 6 <sup>a</sup>  | 87 $\pm$ 6 <sup>ab</sup>  | 79 $\pm$ 10 <sup>ab</sup> | 73 $\pm$ 13 <sup>ab</sup> | 69 $\pm$ 15 <sup>ab</sup> | 64 $\pm$ 21 <sup>ab</sup> | 58 $\pm$ 2 <sup>ab</sup>  | 52 $\pm$ 23 <sup>b</sup>  | 48 $\pm$ 20 <sup>b</sup>  |
| AC           | 96 $\pm$ 3 <sup>a</sup> | 79 $\pm$ 8 <sup>b</sup>  | 72 $\pm$ 12 <sup>b</sup> | 71 $\pm$ 11 <sup>b</sup>  | 69 $\pm$ 12 <sup>b</sup>  | 67 $\pm$ 16 <sup>b</sup>  | 64 $\pm$ 17 <sup>b</sup>  | 60 $\pm$ 21 <sup>b</sup>  | 56 $\pm$ 22 <sup>b</sup>  | 48 $\pm$ 26 <sup>b</sup>  | 47 $\pm$ 26 <sup>b</sup>  |

### Survival through metamorphosis

In experiment 1, a significantly higher proportion of zoeae survived metamorphosis to megalopa in the R+A and R4A treatments than in the R8A and ROT treatments ( $P < 0.05$ ). This was the case irrespective of whether survival was calculated as a percentage of Z1 or Z5 (see methods) (Table 3). Similarly, in experiment 2, percent metamorphosis of both Z1 and Z5 larvae to megalopa was significantly higher in the R+A and R4A treatments than in the R8A, ROT or AC treatments ( $p \leq 0.02$ ).

**Table 3.** Average percent survival through metamorphosis  $\pm$  standard deviation of *Scylla serrata* larvae. M/Z1 = % metamorphosis of zoea 1 to megalopa; M/Z5 = % metamorphosis of zoea 5 to megalopa; R+A = *Artemia* provided on 0 DAH; R4A = *Artemia* provided on 4 DAH; R8A = *Artemia* provided on 8 DAH; ROT = Rotifers only; AC = *Artemia* only. Values in the same column (for each experiment) showing the same superscript are not significantly different. ( $p \leq 0.05$ ;  $n = 9$ ).

| Experiment 1 | Percent survival<br>M/Z1  | Percent survival<br>M/Z5 | Experiment 2 | Percent survival<br>M/Z1 | Percent survival<br>M/Z5 |
|--------------|---------------------------|--------------------------|--------------|--------------------------|--------------------------|
| R+A          | 28 $\pm$ 41 <sup>a</sup>  | 40 $\pm$ 27 <sup>a</sup> | R+A          | 41 $\pm$ 23 <sup>a</sup> | 61 $\pm$ 26 <sup>a</sup> |
| R4A          | 21 $\pm$ 16 <sup>ab</sup> | 35 $\pm$ 16 <sup>a</sup> | R4A          | 37 $\pm$ 17 <sup>a</sup> | 54 $\pm$ 19 <sup>a</sup> |
| R8A          | 3 $\pm$ 3 <sup>b</sup>    | 6 $\pm$ 6 <sup>b</sup>   | R8A          | 8 $\pm$ 8 <sup>b</sup>   | 16 $\pm$ 15 <sup>b</sup> |
| ROT          | 1 $\pm$ 1 <sup>b</sup>    | 2 $\pm$ 2 <sup>b</sup>   | ROT          | 0 $\pm$ 1 <sup>b</sup>   | 1 $\pm$ 1 <sup>b</sup>   |
|              |                           |                          | AC           | 2 $\pm$ 3 <sup>b</sup>   | 3 $\pm$ 1 <sup>b</sup>   |

### Discussion

Considering all parameters, larvae in experiment 2 performed better than those in experiment 1. The two experiments were conducted under identical conditions, suggesting a difference in quality between the two batches of larvae. Differences in quality between batches of *Scylla* larvae have been reported by several authors (Mann *et al.* 1999; Zeng and Li 1999; Millamena and Bangcaya 2001) and it is for this reason that larval rearing experiments should be repeated. However, despite the apparent difference in quality between the two batches, similar trends were observed in the two experiments.

The three parameters that were used to evaluate the effect of feeding schedules on larval performance (zoeal survival, survival through metamorphosis and rate of development), produced contradictory results (Tables 1, 2 and 3 refer). For example, while no significant

differences in survival were measured between zoeae in different treatments up to 9 DAH, there were significant differences between the LSI values. A possible explanation is that nutrition influences development more directly than survival which is influenced by a variety of factors (Anger *et al.* 1981).

Similarly, while no significant differences in survival and development were recorded between the R8A and R+A treatments at 15 DAH, there were significant differences in survival through metamorphosis. Crustacean larvae can often maintain relatively normal growth and survival rates during the zoeal stages despite inadequate nutrition (Sulkin 1978). However the physical changes associated with metamorphosis present a much greater physiological challenge (Forbes and Hay 1988; Charmantier *et al.* 2002). Survival through metamorphosis is thus regarded as the ultimate criterion for evaluating diets for decapod larvae (Sulkin 1978; Harvey 1996; Jeffs *et al.* 1999 ; Ribeiro and Jones 2000). Based on this criterion, larvae in treatments R+A and R4A performed significantly better than those in treatments R8A, ROT and AC in both experiments (Table 3).

The poor performance of the larvae in the AC treatment (Tables 1, 2 and 3) confirmed that the performance of mud crab larvae is compromised when they are not provided with rotifers during their early (Z1 and Z2) life stages (Djunaidah *et al.* 2001a; Mann *et al.* 2001; Quinitio *et al.* 2001; Hamasaki *et al.* 2002b). It has been previously demonstrated that mudcrab larvae can be reared on a diet of *Artemia* nauplii only (Ong 1966; Du Plessis 1971; Brick 1974; Heasman and Fielder 1983; Fielder *et al.* 1988; Mann and Parlato 1995; Baylon *et al.* 2001a; Nghia *et al.* 2001b; Suprayudi *et al.* 2002a). However, early stage mud crab larvae are considered to be inefficient predators that capture food opportunistically (Heasman and Fielder 1983; Zeng and Li 1999). Moreover, *Artemia* nauplii are large (428-517 µm body length) in relation to Z1 mud crab larvae (810±220 µm lateral spine width) and swim vigorously, making it difficult for the larvae to catch and handle them (Léger *et al.* 1986; Van Stappen 1996; Harvey and Epifanio 1997). Rotifers are much smaller (45-180 µm body length), swim more slowly, and are therefore easier for crab larvae to catch and handle (Dhert 1996). It is therefore suggested that the larvae in treatment AC were not able to consume sufficient numbers of prey during their early life stages and that this compromised growth and survival throughout the rearing period.

Larvae in the ROT treatment also performed poorly, particularly during the latter stages of development (Tables 1, 2 and 3). Although rotifers are generally considered to be a more suitable live food for the early larval stages of *Scylla serrata*, this provides further evidence

that the diet must be supplemented with *Artemia* (Takeuchi *et al.* 2000; Baylon *et al.* 2001a; Suprayudi *et al.* 2002a). The energy content of individual rotifers is low and mud crab larvae are only capable of consuming a limited quantity within a given time frame (maximum 3-10 per hour) (Lumasag and Quinitio 1998). As larvae grow the cost : benefit ratio of eating a small food item (such as rotifers) worsens (Von Herbing *et al.* 2001; Bolnick and Ferry-Graham 2002). The point at which rotifers become inadequate as a sole food source for crustacean larvae can therefore be quantified (Emmerson 1984; Zeng and Li 1999).

The poor performance of larvae in treatment R8A suggests that *Artemia* should be introduced before the end of the Z3 stage. The Z3 stage has been identified as a critical stage of nutritional vulnerability beyond which deprivation of a suitable food organisms compromises survival and development in *S. paramamosain* (Li *et al.* 1998; Zeng and Li 1999), *S. tranquebarica* (Takeuchi *et al.* 2000), *S. serrata* (Suprayadi *et al.* 2002) and other crab larvae (Sulkin 1978). Crab larvae become more efficient and selective predators during their later life stages (Z3 onwards in the case of *Scylla*), and show a strong preference for *Artemia* over rotifers (Harvey and Epifanio 1997; Zeng 1998).

The best survival, growth and metamorphosis were recorded for larvae in treatments R+A and R4A (Tables 1,2 and 3). This confirms the observations by Baylon *et al.* (2001) that early supplementation of the diet with *Artemia* nauplii enhances the performance of *Scylla serrata* larvae. Whereas the weight specific energy content of *Artemia* and rotifers is virtually identical ( $\pm 0.02$  J/ $\mu$ g), *Artemia* nauplii (2.7  $\mu$ g) are considerably heavier than rotifers (0.5  $\mu$ g) (Léger *et al.* 1986; Harvey and Epifanio 1997). In the presence of equal numbers of rotifers and *Artemia* nauplii, significantly more of the former were caught and consumed by early stage *Panopeus herbstii* zoea. Despite this, *Artemia* accounted for the bulk of energy intake (Harvey and Epifanio 1997). As Merchie (1996) points out, larval predators expend less energy fulfilling their energetic requirements when consuming larger food items (such as *Artemia*) that have high individual energy content. Thus, even if relatively few *Artemia* nauplii were caught by the early stage zoeae in the two experiments, they would have contributed the bulk of nutrients (including energy) which manifested itself in better performance throughout the rearing period.

On the other hand Suprayudi *et al.* (2002a) found that supplementing the larval diet of Japanese *Scylla serrata* with *Artemia* before the Z3 stage did not enhance survival, development or metamorphosis. This suggests that early stage South African *Scylla serrata* larvae are more adept at feeding on *Artemia* nauplii. *Artemia* densities in our study (10/ml)

were however higher than those used by Suprayudi *et al.* (2002a) (4/ml), which may have ensured that more *Artemia* were caught by the early zoea. This may also explain why Suprayadi *et al.* (2002) observed cannibalism during the zoeal stages, whereas in our study only megalopae demonstrated this behaviour.

In conclusion the present study has shown that the zoeae of South African *S. serrata* larvae require rotifers as a first food and that considerable benefit is gained by the introduction of *Artemia* nauplii from the Z1 stage. However, given several zootechnical and nutritional problems, the addition of *Artemia* at the Z1 stage under pilot or commercial scale hatchery conditions, would not be practical. For instance larvae cannot be transferred to clean vessels daily. Moreover if rotifers and *Artemia* are not continually enriched with micro algae or enrichment media, their nutritional value is depleted within a few hours (Dhert 1996; Merchie 1996). It is, therefore, desirable to flush uneaten live food from the culture tanks on a daily basis. However even in the absence of micro algae *Artemia* nauplii quickly grow larger than *S. serrata* Z1 larvae and it therefore becomes impossible to flush them out without losing larvae through the appropriately sized outlet screens in the process. It is only at 4 DAH when the majority of the larvae have moulted to Z2 that they are large enough to be retained by plankton mesh large enough to allow uneaten, 24 hour old *Artemia* metanauplii to be flushed out. Given that there were no significant differences in growth, survival or metamorphosis between the R+A and R4A treatments suggests that *Artemia* should be introduced at 3 to 4 DAH under large scale conditions. This corresponds to the beginning of the second zoeal stage. *Artemia* supplementation should however not be delayed beyond the Z3 stage.

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# Chapter 7

## **The effect of fatty acid enrichment on growth, survival and metamorphosis of South African mud crab *Scylla serrata* (Forskål) larvae**

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## Abstract

Little is known about the nutritional requirements of *Scylla serrata* larvae. To determine whether boosting the live food with highly unsaturated fatty acids (HUFA) could improve survival and development, four experiments, using larvae from four different female crabs, were conducted over approximately six months.

In the first two experiments larvae were fed *Artemia* throughout the rearing period and in the second two, larvae were fed rotifers for the first eight days and *Artemia* nauplii from day six onwards. Larvae in the control groups of all four experiments were fed unenriched live food. In the first two experiments, the performance of larvae in the control was compared with larvae fed *Artemia* enriched with algae and two different commercial emulsions. In the third experiment larvae in the three treatments were fed unenriched rotifers and then *Artemia* enriched with three experimental emulsions containing different levels of (n-3) HUFA. In the fourth experiment, larvae in the treatment groups were fed either enriched rotifers (commercial emulsion), or enriched *Artemia* or both enriched rotifers and enriched *Artemia*.

No significant differences in development or survival were recorded for larvae in any of the treatments in the first three experiments. In the fourth experiment, performance of the larvae was significantly improved only when both rotifers and *Artemia* were enriched.

The results suggest that enriching only the *Artemia* has little benefit for the larvae. In order for enrichment to be effective, both the rotifers and the *Artemia* must be enriched with HUFA.

## Introduction

*Scylla serrata* is a potential aquaculture species in South Africa due to its fast growth rate, high market price and limited potential for commercial harvesting from the wild (Robertson and Kruger 1994; Robertson 1996; Williams and Primavera 2001). One of the major bottlenecks to the establishment of commercial farming is a lack of hatchery produced seed. Larval cultures are characterized by low and variable survival, particularly toward the end of the rearing period. Mass mortality at metamorphosis from the fifth zoeal stage (Z5) to megalopa is so common that it is referred to as moult death syndrome (MDS). It has been suggested that MDS is caused by inferior nutrition during larval rearing (Li *et al.* 1999; Zeng and Li 1999; Mann *et al.* 2001; Marichamy and Rajapackiam 2001; Qunitio *et al.* 2001; Hamasaki *et al.* 2002a, b; Suprayudi *et al.* 2002b).

It is generally accepted that mud crab larvae in the wild feed on marine zooplankton. By feeding on phytoplankton, zooplankton accumulate large reserves of wax esters which are rich in highly unsaturated fatty acids (HUFA) (Sargent *et al.* 1990). This ensures that the full range of fatty acids necessary for metabolic energy and cell membrane development of larvae in the wild is provided by the diet. Mud crab larvae are usually cultured on a combination of rotifers (usually *Brachionus* spp.) and *Artemia* nauplii (Takeuchi *et al.* 2000; Djunaidah *et al.* 2001a; Mann *et al.* 2001; Quintio *et al.* 2001; Hamasaki *et al.* 2002b; Suprayudi *et al.* 2002a). Whilst these live foods satisfy the protein and mineral requirements for most marine species they often lack certain essential fatty acids (Watanabe *et al.* 1980). Rotifers and *Artemia* contain short chained, saturated fatty acids such as linoleic (18: 2(n-6)) and linolenic (18: 3(n-3) acids). However, marine crustaceans have a limited ability to elongate and desaturate them (Kanazawa *et al.* 1979a; Teshima *et al.* 1992; Merican and Shim 1996). They also cannot synthesize (n-3) and (n-6) families of fatty acids *de novo* and therefore require adequate quantities of these essential fatty acids (EFA) to be added to the diet (Kanazawa *et al.* 1979a; González-Félix *et al.* 2002a).

The survival, growth and resistance to stress of several marine crustacean species have been enhanced by boosting the diet with EFAs (Xu *et al.* 1993; Rees *et al.* 1994; González-Félix 2002b). Of the HUFAs, members of the (n-3) and (n-6) family, especially eicosapentaenoic acid (EPA) (20:5(n-3)) (Glencross and Smith 2001a; González-Félix *et al.* 2002b), docosahexaenoic acid (DHA) (22:6(n-3)) (Kanazawa *et al.* 1979b; González-Félix *et al.* 2002b) and arachidonic acid (ARA) (20:4(n-6)) (Glencross and Smith 2001b) have been found to be EFAs for the larvae of many crustacean species.

Little is known about the particular EFA and (n-3) HUFA requirements of even penaeid shrimp, the most extensively cultured crustaceans (Rees *et al.* 1994; González-Félix *et al.* 2002a). Researching EFA requirements for mud crab is further complicated since the larvae cannot be reared on inert diets exclusively (Quintio *et al.* 1999). Nevertheless, several authors have reported improvements in larval performance of mud crab and other portunids (e.g. *Portunus trituberculatus*) after boosting the levels of (n-3) HUFA (including EPA and DHA) in the live food (Takeuchi *et al.* 1999; Kobayashi *et al.* 2000; Djunaidah *et al.* 2001a; Hamasaki *et al.* 2002a; Suprayudi *et al.* 2002a, b). However Mann *et al.* (2001) found that enriching *Artemia* with commercial enrichment media had no significant effect on the performance of *S. serrata* larvae in Australia. The fatty acid requirements of crustacean larvae also often differ between species of the same genus, and within species requirements may differ between populations. This study was conducted to determine whether the performance of

South African *Scylla serrata* larvae could be enhanced by boosting the levels of (n-3) HUFA in the live food.

## Materials and methods

### *Systems and procedures*

The study was carried out at Mtunzini Prawn Farm on the subtropical east coast of South Africa. The experiments were undertaken in an insulated, air conditioned laboratory. Identical 2 L cylindro-conical plastic jars were used as rearing containers. Filtered (1  $\mu$ m) seawater (32 g/L) was used in all trials and was replaced daily. Temperature was maintained at 29-30°C by standing the jars in a heated water bath. The seawater was treated with 5 mg/L oxytetracycline-hydrochloride daily to prevent microbial infections. The water was aerated using open ended plastic air tubes. Photoperiod was maintained at 16L: 8D with white fluorescent tubes.

Broodstock females were caught in the Umlalazi estuary and matured in a black, bare bottomed, 5x4x1 m deep concrete tank at a density of 0.5 crabs/m<sup>2</sup>. The temperature of the seawater fluctuated between 19 and 25 °C and was recirculated through a biofilter. Subdued light was provided on a 14L : 10D photoperiod. Crabs were fed *ad libitum* on fish roe, mussel, shrimp or chopped squid on successive days. Uneaten food and faeces were siphoned from the maturation tank daily. Females extruded eggs in sand filled containers that were placed on the bottom of the maturation tank. Berried crabs were transferred to a 60 L glass aquarium for observation and prophylactic treatment of the eggs, whereafter they were transferred into black, 600 L, covered fibreglass tanks provided with recirculated, UV sterilised seawater at 27°C. Larvae generally hatched 288 hours after spawning. Detailed information on broodstock maintenance and spawning is provided in Davis *et al.* (2003a).

One hour after hatching, positively photo-tactic larvae were individually selected with a wide bore pipette and stocked at a density of 67/L in the rearing containers (120 larvae per vessel). Each day, the contents of the rearing containers was gently poured into a 3 L bowl and the surviving larvae counted while being pipetted into new containers containing fresh seawater. The “new” containers were incubated beforehand in the same water bath as the “old” rearing vessels to equilibrate temperature. Ammonia (NH<sub>4</sub><sup>+</sup>-N) was maintained at 0 - 0.05 mg/L; nitrite (NO<sub>2</sub>- N) at 0 - 0.05 mg/L; pH at 8.0 - 8.6 and oxygen at 8.0 - 8.2 mg/L.

Four experiments were conducted over a six month period. Each experiment used larvae from a different female crab. Each experiment had four treatments. There were nine replicates per treatment. The nine replicates were divided into three groups of three containers each. The position of these groups was randomised within the experimental grid.

Development was monitored every three days by identifying the zoeal instar stage of each larva and assigning a value: first zoea (Z1) =1; second zoea (Z2) =2, etc. to megalopa (M) = 6. To compare development in each treatment an average larval stage index (LSI) ( $n = 9$ ) was calculated as described by Millamena and Bangcaya (2001).

Z5 first moulted (metamorphosed) into megalopae 15 days after hatch (DAH) and were removed from the system to prevent cannibalism. Survival of the zoeae is thus presented until 15 DAH. The number of megalopae produced within each treatment group was totalled at 18 DAH. Survival to megalopa (%) was measured in two ways: firstly as the number of Z1 surviving to megalopa ( $M/Z1 \times 100$ ) and secondly as the number of Z5 surviving the metamorphosis to megalopa ( $M/Z5 \times 100$ ).

### *Food and Feeding*

Rotifers (*Brachionus plicatilis*) were cultured outdoors in a 2600 L semi-continuous recirculating system and fed a mixture of *Chlorella* sp., baker's yeast and Culture Selco® (INVE Aquaculture, Belgium). They were harvested daily through a 60  $\mu$ m screen, and rinsed with clean seawater. Where appropriate, rotifers were enriched according to Dhert (1996). Both enriched and unenriched rotifers were rinsed with seawater that was slowly heated to 29°C to avoid exposing them to thermal shock when they were added to the larval rearing vessels.

*Artemia* nauplii (EG® type, INVE Aquaculture, Belgium) were hatched and enriched as described by Van Stappen (1996). In situations where, after harvesting, they could not immediately be fed to the larvae, both enriched metanauplii and newly hatched nauplii were kept at  $\pm 5^\circ\text{C}$  to delay development and prevent loss of nutrients (Merchie 1996). *Artemia* were then rinsed with tap water and suspended at a known density in 29°C seawater.

Rotifers and *Artemia* were provided at higher than normal densities: 40/mL and 15/mL respectively (Baylon *et al.* 2001a; Quintio *et al.* 2001; Wickins and Lee 2002) to ensure that access to prey was not a limiting factor. The larvae were fed once daily at 09:00 in the morning.

Figure 1 illustrates the feeding regimes. In the first two experiments (1 and 2), larvae were fed only *Artemia*. In the control groups the *Artemia* were unenriched (Fig. 1). Larvae in the treatment groups were provided with unenriched *Artemia* nauplii from hatch until 6 DAH and enriched *Artemia* from 7 DAH to 18 DAH. In experiment 1, *Artemia* were enriched with either *Tetraselmis* algae (treatment ALG), Super Selco® (INVE Aquaculture, Belgium) (treatment SS) or DHA Selco® (INVE Aquaculture, Belgium) (treatment DHA). In experiment 2, *Artemia* were enriched with *Tetraselmis* (treatment ALG), DHA Selco® (treatment DHA) or 0% ICES (International Council for the Exploration of the Sea) emulsion (treatment ZERO).

In experiment 3, larvae were fed unenriched rotifers from hatch until 8 DAH and *Artemia* from 6 DAH until 18 DAH. In the three treatment groups, the *Artemia* were enriched with ICES emulsions containing 0%, 30%, and 50% (n-3) HUFA respectively. The DHA/EPA ratio of the emulsions was 0.6. In the control the *Artemia* nauplii were not enriched.

In experiment 4, larvae were fed rotifers from hatch until 8 DAH and *Artemia* from 6 DAH onwards. In the first treatment only the rotifers were enriched with Super Selco® and the *Artemia* nauplii were unenriched (treatment ER); in the second treatment, unenriched rotifers and *Artemia* enriched with Super Selco® were fed (treatment EA); in the third treatment, both rotifers and *Artemia* were enriched (ER+EA); and in the control group, neither rotifers, nor *Artemia* were enriched.

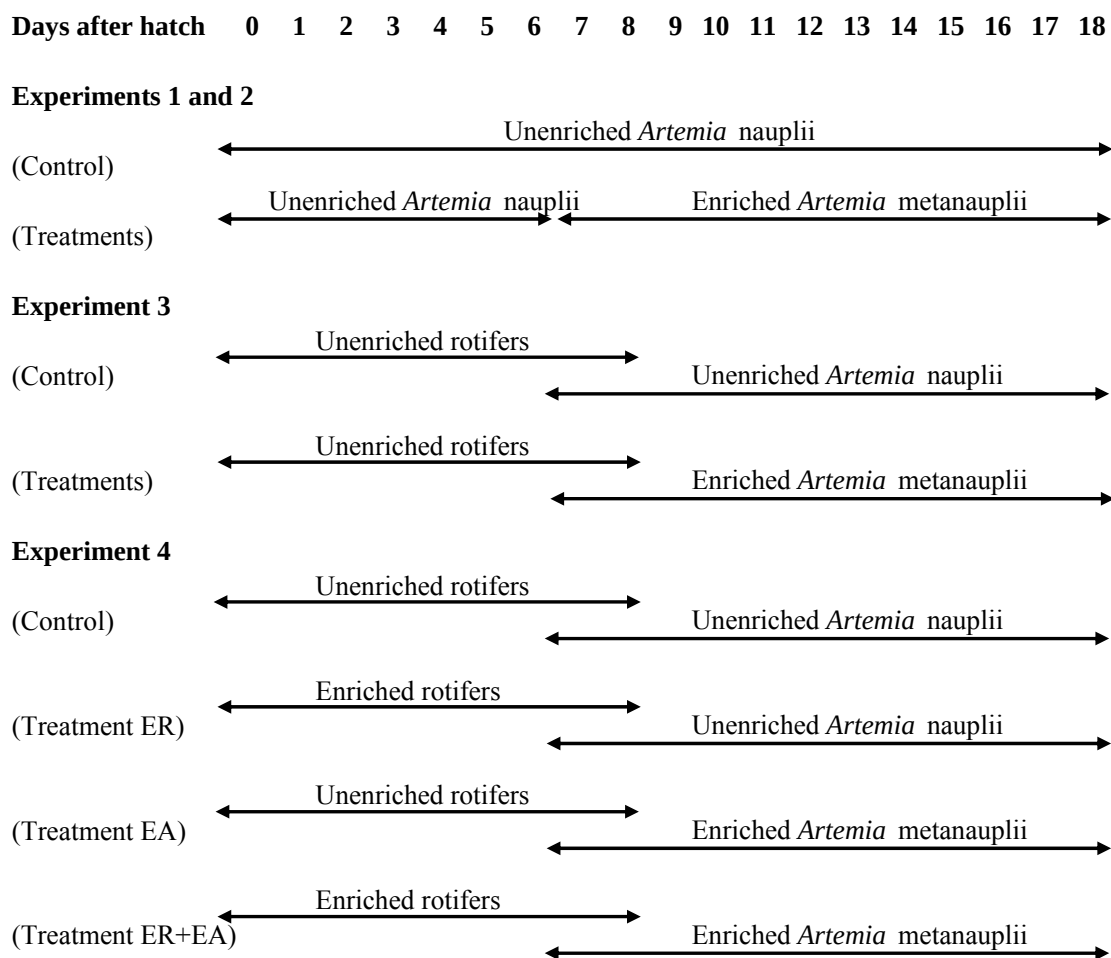


**Figure 1.** The feeding schedules for the live food enrichment experiments.

### *Fatty acid analysis*

Rotifers and *Artemia* were sampled on three separate occasions both before and after enrichment, rinsed and freeze dried for storage and transport. The three samples were pooled before analysis.

In experiments 1 and 4, Z1 larvae were randomly sampled from the hatching tank before the experiment. Zoea 5 larvae from three of the nine replicates of each treatment were sacrificed at 15 DAH. In addition, Z1 larvae from 16 other batches produced by different female crabs over an 18 month period were similarly sampled. The larvae were rinsed and freeze dried before analysis.

To determine the levels of HUFA contained in the lipid portion of the tissue, the live food and the larvae were submitted to fatty acid methyl ester (FAME) analysis at the Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Belgium. The

analysis - a modified procedure of Lepage and Roy (1984) was conducted as described by Coutteau and Sorgeloos (1995).

### *Statistical analyses*

Homogeneity of variance for LSI values, percent survival and percent metamorphosis was tested with the Levene statistic. If no significant differences were detected between the variances, the data were submitted to a one-way ANOVA. The Tukey HSD post-hoc analysis was used to detect differences between the means and to indicate areas of significant difference. If significant differences were detected between variances, the data were transformed using either logarithmic or arcsine-square root transformations. If heterogeneity was still detected, non parametric analyses were used to detect significant differences between means (Sokal and Rohlf 1995). Kruskal-Wallis H was used to detect whether there were significant differences between the means and a multiple comparison of mean ranks for all groups was used to identify areas of significant difference (Lehmann 1998). The relationship between LSI at 15 DAH, survival at 15 DAH or % survival to megalopa was compared with FAME levels in the Z5 larvae just prior to metamorphosis using a regression analysis (Sokal and Rohlf 1995).

## **Results**

### *Larval development*

In experiments 1, 2 and 3, no significant differences in LSI value were recorded at any stage of development in any of the treatments (Table 1).

In experiment 4, there were no significant differences in larval development rates between any of the treatments up to 3 DAH. At 6 and 9 DAH, significantly higher LSI values were recorded for larvae fed enriched rotifers (in treatments ER and ER+EA) than for those fed unenriched rotifers (in the control and treatment EA) ( $p \leq 0.04$ ). At 12 and 15 DAH, LSI values for larvae in treatment ER+EA were significantly higher than those in the control ( $p \leq 0.05$ ). LSI values for larvae in treatments ER and EA were intermediate.

**Table 1.** Average larval stage index (LSI) values  $\pm$  standard deviation between 3 and 15 days after hatch of *Scylla serrata* larvae fed different enrichment diets. Control = no enrichment; ALG = *Artemia* enriched with *Tetraselmis*; SS = *Artemia* enriched with Super Selco®; DHA = *Artemia* enriched with DHA Selco®; ZERO = *Artemia* enriched with 0% (n-3) HUFA; THIRTY = *Artemia* enriched with 30% (n-3) HUFA; FIFTY = *Artemia* enriched with 50% (n-3) HUFA; ER = only rotifers enriched with Super Selco®; EA = only *Artemia* enriched with Super Selco®; ER+EA = both rotifers and *Artemia* enriched with Super Selco®. Values in the same column (for each experiment) showing the same superscript are not significantly different. ( $p \leq 0.05$ ;  $n = 9$ ).

| Treatment    | Day3                         | Day6                         | Day9                         | Day12                         | Day15                         |
|--------------|------------------------------|------------------------------|------------------------------|-------------------------------|-------------------------------|
| Experiment 1 |                              |                              |                              |                               |                               |
| Control      | 1.57 $\pm$ 0.08 <sup>a</sup> | 2.22 $\pm$ 0.62 <sup>a</sup> | 3.49 $\pm$ 0.32 <sup>a</sup> | 4.68 $\pm$ 0.40 <sup>a</sup>  | 5.27 $\pm$ 0.48 <sup>a</sup>  |
| ALG          | 1.68 $\pm$ 0.05 <sup>a</sup> | 2.35 $\pm$ 0.56 <sup>a</sup> | 3.42 $\pm$ 0.49 <sup>a</sup> | 4.05 $\pm$ 0.85 <sup>a</sup>  | 4.80 $\pm$ 0.81 <sup>a</sup>  |
| SS           | 1.42 $\pm$ 0.17 <sup>a</sup> | 2.11 $\pm$ 0.49 <sup>a</sup> | 3.56 $\pm$ 0.50 <sup>a</sup> | 4.42 $\pm$ 0.67 <sup>a</sup>  | 5.15 $\pm$ 0.55 <sup>a</sup>  |
| DHA          | 1.81 $\pm$ 0.09 <sup>a</sup> | 2.28 $\pm$ 0.51 <sup>a</sup> | 3.64 $\pm$ 0.33 <sup>a</sup> | 4.63 $\pm$ 0.29 <sup>a</sup>  | 5.10 $\pm$ 0.41 <sup>a</sup>  |
| Experiment 2 |                              |                              |                              |                               |                               |
| Control      | 1.43 $\pm$ 0.18 <sup>a</sup> | 2.46 $\pm$ 0.24 <sup>a</sup> | 3.64 $\pm$ 0.35 <sup>a</sup> | 4.77 $\pm$ 0.15 <sup>a</sup>  | 5.16 $\pm$ 0.49 <sup>a</sup>  |
| ALG          | 1.43 $\pm$ 0.17 <sup>a</sup> | 2.53 $\pm$ 0.27 <sup>a</sup> | 3.68 $\pm$ 0.39 <sup>a</sup> | 4.49 $\pm$ 0.58 <sup>a</sup>  | 4.86 $\pm$ 0.40 <sup>a</sup>  |
| ZERO         | 1.89 $\pm$ 0.04 <sup>a</sup> | 2.75 $\pm$ 0.16 <sup>a</sup> | 3.81 $\pm$ 0.20 <sup>a</sup> | 4.76 $\pm$ 0.22 <sup>a</sup>  | 5.11 $\pm$ 0.25 <sup>a</sup>  |
| DHA          | 1.75 $\pm$ 0.15 <sup>a</sup> | 2.70 $\pm$ 0.14 <sup>a</sup> | 3.27 $\pm$ 0.56 <sup>a</sup> | 4.27 $\pm$ 0.58 <sup>a</sup>  | 4.72 $\pm$ 0.63 <sup>a</sup>  |
| Experiment 3 |                              |                              |                              |                               |                               |
| Control      | 1.82 $\pm$ 0.07 <sup>a</sup> | 2.69 $\pm$ 0.35 <sup>a</sup> | 3.74 $\pm$ 0.18 <sup>a</sup> | 4.16 $\pm$ 0.40 <sup>a</sup>  | 4.85 $\pm$ 0.28 <sup>a</sup>  |
| ZERO         | 1.79 $\pm$ 0.09 <sup>a</sup> | 2.62 $\pm$ 0.16 <sup>a</sup> | 3.52 $\pm$ 0.30 <sup>a</sup> | 4.31 $\pm$ 0.44 <sup>a</sup>  | 4.87 $\pm$ 0.28 <sup>a</sup>  |
| THIRTY       | 1.88 $\pm$ 0.08 <sup>a</sup> | 2.58 $\pm$ 0.30 <sup>a</sup> | 3.64 $\pm$ 0.23 <sup>a</sup> | 4.08 $\pm$ 0.58 <sup>a</sup>  | 4.78 $\pm$ 0.19 <sup>a</sup>  |
| FIFTY        | 1.90 $\pm$ 0.04 <sup>a</sup> | 2.80 $\pm$ 0.19 <sup>a</sup> | 3.77 $\pm$ 0.14 <sup>a</sup> | 4.30 $\pm$ 0.34 <sup>a</sup>  | 4.85 $\pm$ 0.26 <sup>a</sup>  |
| Experiment 4 |                              |                              |                              |                               |                               |
| Control      | 1.83 $\pm$ 0.05 <sup>a</sup> | 2.38 $\pm$ 0.17 <sup>b</sup> | 2.80 $\pm$ 0.42 <sup>b</sup> | 3.96 $\pm$ 0.47 <sup>b</sup>  | 4.27 $\pm$ 0.52 <sup>b</sup>  |
| ER           | 1.92 $\pm$ 0.03 <sup>a</sup> | 2.55 $\pm$ 0.10 <sup>a</sup> | 3.46 $\pm$ 0.36 <sup>a</sup> | 4.47 $\pm$ 0.30 <sup>ab</sup> | 4.96 $\pm$ 0.63 <sup>ab</sup> |
| EA           | 1.88 $\pm$ 0.08 <sup>a</sup> | 2.38 $\pm$ 0.21 <sup>b</sup> | 3.03 $\pm$ 0.27 <sup>b</sup> | 4.21 $\pm$ 0.28 <sup>ab</sup> | 4.61 $\pm$ 0.49 <sup>ab</sup> |
| ER+EA        | 1.68 $\pm$ 0.02 <sup>a</sup> | 2.58 $\pm$ 0.11 <sup>a</sup> | 3.44 $\pm$ 0.19 <sup>a</sup> | 4.61 $\pm$ 0.19 <sup>a</sup>  | 5.30 $\pm$ 0.31 <sup>a</sup>  |

*Zoeal survival*

In experiments 1, 2 and 3 no significant differences in survival were detected between larvae in any of the treatments at any zoeal stage (Table 2).

In experiment 4, no difference in survival was recorded for zoeae in any of the treatments during the first 6 DAH. At 7 DAH high mortality was recorded for larvae receiving unenriched rotifers (treatments EA and Control) relative to those receiving enriched rotifers (treatments ER and ER+EA) which resulted in a significantly higher survival of larvae in treatment ER+EA than those in the control. Similarly high mortality in the control was recorded at 8 and 9 DAH. Although this did not result in statistically significant differences in survival of the zoeae between any of the four treatments up till 14 DAH, Figure 2 clearly illustrates the difference in zoeal performance between the treatments fed enriched as opposed to unenriched rotifers. By 15 DAH, significantly higher survival was recorded for zoeae in treatment ER+EA than for those in the control treatment ( $p = 0.04$ ).

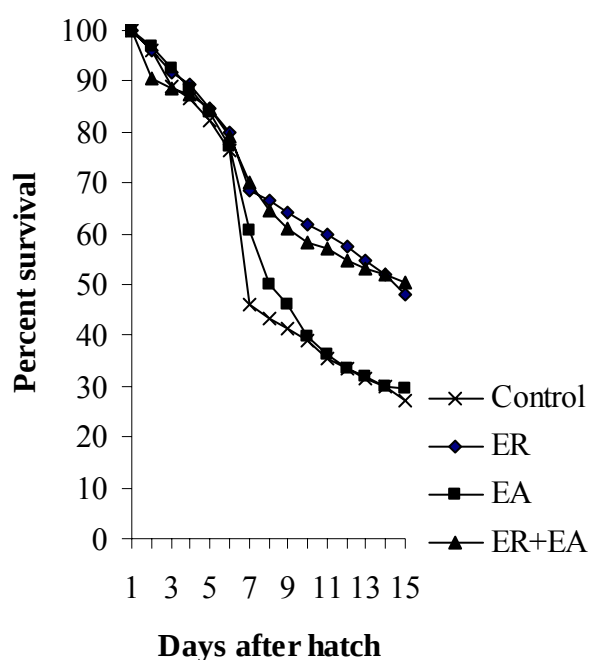


**Figure 2.** Survival of *Scylla serrata* zoeae in experiment 3. Control = neither rotifers nor *Artemia* enriched. ER = only rotifers enriched with Super Selco®; EA = only *Artemia* enriched with Super Selco®; ER+EA = both rotifers and *Artemia* enriched with Super Selco®.

**Table 2.** Average percent survival  $\pm$  standard deviation of *Scylla serrata* zoeae fed different enrichment diets between 3 and 15 days after hatch. Control = no enrichment; ALG = *Artemia* enriched with *Tetraselmis*; SS = *Artemia* enriched with Super Selco® (SS); DHA = *Artemia* enriched with DHA Selco®; ZERO = *Artemia* enriched with 0% (n-3) HUFA; THIRTY = *Artemia* enriched with 30% (n-3) HUFA; FIFTY = *Artemia* enriched with 50% (n-3) HUFA; ER = only rotifers enriched with SS; EA = only *Artemia* enriched with SS; ER+EA = both rotifers and *Artemia* enriched with SS. Values in the same column (for each experiment) showing the same superscript are not significantly different. ( $p \leq 0.05$ ;  $n = 9$ ).

| Treatment    | Day3                     | Day6                     | Day7                      | Day8                     | Day9                     | Day10                    | Day11                    | Day12                    | Day13                    | Day14                    | Day15                     |
|--------------|--------------------------|--------------------------|---------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|---------------------------|
| Experiment 1 |                          |                          |                           |                          |                          |                          |                          |                          |                          |                          |                           |
| Control      | 94 $\pm$ 3 <sup>a</sup>  | 84 $\pm$ 14 <sup>a</sup> | 81 $\pm$ 16 <sup>a</sup>  | 80 $\pm$ 14 <sup>a</sup> | 77 $\pm$ 17 <sup>a</sup> | 72 $\pm$ 20 <sup>a</sup> | 69 $\pm$ 20 <sup>a</sup> | 67 $\pm$ 20 <sup>a</sup> | 53 $\pm$ 21 <sup>a</sup> | 50 $\pm$ 22 <sup>a</sup> | 39 $\pm$ 24 <sup>a</sup>  |
| ALG          | 95 $\pm$ 2 <sup>a</sup>  | 89 $\pm$ 3 <sup>a</sup>  | 85 $\pm$ 5 <sup>a</sup>   | 84 $\pm$ 5 <sup>a</sup>  | 80 $\pm$ 6 <sup>a</sup>  | 78 $\pm$ 7 <sup>a</sup>  | 74 $\pm$ 9 <sup>a</sup>  | 70 $\pm$ 13 <sup>a</sup> | 66 $\pm$ 13 <sup>a</sup> | 60 $\pm$ 15 <sup>a</sup> | 44 $\pm$ 21 <sup>a</sup>  |
| SS           | 95 $\pm$ 3 <sup>a</sup>  | 88 $\pm$ 4 <sup>a</sup>  | 87 $\pm$ 5 <sup>a</sup>   | 84 $\pm$ 4 <sup>a</sup>  | 83 $\pm$ 4 <sup>a</sup>  | 81 $\pm$ 5 <sup>a</sup>  | 78 $\pm$ 7 <sup>a</sup>  | 75 $\pm$ 8 <sup>a</sup>  | 74 $\pm$ 8 <sup>a</sup>  | 69 $\pm$ 8 <sup>a</sup>  | 49 $\pm$ 20 <sup>a</sup>  |
| DHA          | 96 $\pm$ 3 <sup>a</sup>  | 92 $\pm$ 3 <sup>a</sup>  | 83 $\pm$ 18 <sup>a</sup>  | 81 $\pm$ 17 <sup>a</sup> | 78 $\pm$ 18 <sup>a</sup> | 78 $\pm$ 17 <sup>a</sup> | 76 $\pm$ 16 <sup>a</sup> | 74 $\pm$ 17 <sup>a</sup> | 71 $\pm$ 16 <sup>a</sup> | 63 $\pm$ 16 <sup>a</sup> | 56 $\pm$ 18 <sup>a</sup>  |
| Experiment 2 |                          |                          |                           |                          |                          |                          |                          |                          |                          |                          |                           |
| Control      | 92 $\pm$ 4 <sup>a</sup>  | 81 $\pm$ 10 <sup>a</sup> | 77 $\pm$ 13 <sup>a</sup>  | 73 $\pm$ 18 <sup>a</sup> | 70 $\pm$ 18 <sup>a</sup> | 68 $\pm$ 18 <sup>a</sup> | 66 $\pm$ 19 <sup>a</sup> | 64 $\pm$ 19 <sup>a</sup> | 62 $\pm$ 20 <sup>a</sup> | 55 $\pm$ 19 <sup>a</sup> | 36 $\pm$ 19 <sup>a</sup>  |
| ALG          | 91 $\pm$ 10 <sup>a</sup> | 82 $\pm$ 15 <sup>a</sup> | 81 $\pm$ 14 <sup>a</sup>  | 78 $\pm$ 16 <sup>a</sup> | 76 $\pm$ 17 <sup>a</sup> | 74 $\pm$ 17 <sup>a</sup> | 72 $\pm$ 17 <sup>a</sup> | 68 $\pm$ 21 <sup>a</sup> | 66 $\pm$ 19 <sup>a</sup> | 61 $\pm$ 19 <sup>a</sup> | 52 $\pm$ 18 <sup>a</sup>  |
| DHA          | 88 $\pm$ 14 <sup>a</sup> | 77 $\pm$ 14 <sup>a</sup> | 73 $\pm$ 14 <sup>a</sup>  | 69 $\pm$ 14 <sup>a</sup> | 66 $\pm$ 14 <sup>a</sup> | 64 $\pm$ 13 <sup>a</sup> | 60 $\pm$ 14 <sup>a</sup> | 53 $\pm$ 19 <sup>a</sup> | 47 $\pm$ 20 <sup>a</sup> | 45 $\pm$ 26 <sup>a</sup> | 19 $\pm$ 4 <sup>a</sup>   |
| ZERO         | 94 $\pm$ 2 <sup>a</sup>  | 86 $\pm$ 3 <sup>a</sup>  | 83 $\pm$ 4 <sup>a</sup>   | 81 $\pm$ 4 <sup>a</sup>  | 77 $\pm$ 6 <sup>a</sup>  | 76 $\pm$ 6 <sup>a</sup>  | 75 $\pm$ 7 <sup>a</sup>  | 70 $\pm$ 9 <sup>a</sup>  | 67 $\pm$ 11 <sup>a</sup> | 61 $\pm$ 8 <sup>a</sup>  | 36 $\pm$ 14 <sup>a</sup>  |
| Experiment 3 |                          |                          |                           |                          |                          |                          |                          |                          |                          |                          |                           |
| Control      | 85 $\pm$ 25 <sup>a</sup> | 77 $\pm$ 24 <sup>a</sup> | 73 $\pm$ 24 <sup>a</sup>  | 72 $\pm$ 24 <sup>a</sup> | 69 $\pm$ 24 <sup>a</sup> | 67 $\pm$ 24 <sup>a</sup> | 64 $\pm$ 24 <sup>a</sup> | 60 $\pm$ 24 <sup>a</sup> | 57 $\pm$ 25 <sup>a</sup> | 56 $\pm$ 26 <sup>a</sup> | 51 $\pm$ 24 <sup>a</sup>  |
| ZERO         | 97 $\pm$ 3 <sup>a</sup>  | 85 $\pm$ 5 <sup>a</sup>  | 81 $\pm$ 7 <sup>a</sup>   | 80 $\pm$ 8 <sup>a</sup>  | 76 $\pm$ 7 <sup>a</sup>  | 71 $\pm$ 11 <sup>a</sup> | 68 $\pm$ 13 <sup>a</sup> | 63 $\pm$ 15 <sup>a</sup> | 60 $\pm$ 17 <sup>a</sup> | 57 $\pm$ 19 <sup>a</sup> | 51 $\pm$ 19 <sup>a</sup>  |
| THIRTY       | 95 $\pm$ 2 <sup>a</sup>  | 77 $\pm$ 19 <sup>a</sup> | 73 $\pm$ 20 <sup>a</sup>  | 71 $\pm$ 21 <sup>a</sup> | 67 $\pm$ 23 <sup>a</sup> | 62 $\pm$ 28 <sup>a</sup> | 60 $\pm$ 29 <sup>a</sup> | 56 $\pm$ 29 <sup>a</sup> | 53 $\pm$ 29 <sup>a</sup> | 50 $\pm$ 28 <sup>a</sup> | 48 $\pm$ 27 <sup>a</sup>  |
| FIFTY        | 97 $\pm$ 4 <sup>a</sup>  | 70 $\pm$ 34 <sup>a</sup> | 68 $\pm$ 34 <sup>a</sup>  | 67 $\pm$ 34 <sup>a</sup> | 65 $\pm$ 33 <sup>a</sup> | 62 $\pm$ 33 <sup>a</sup> | 60 $\pm$ 32 <sup>a</sup> | 52 $\pm$ 34 <sup>a</sup> | 50 $\pm$ 33 <sup>a</sup> | 47 $\pm$ 32 <sup>a</sup> | 42 $\pm$ 29 <sup>a</sup>  |
| Experiment 4 |                          |                          |                           |                          |                          |                          |                          |                          |                          |                          |                           |
| Control      | 89 $\pm$ 8 <sup>a</sup>  | 76 $\pm$ 8 <sup>a</sup>  | 46 $\pm$ 19 <sup>b</sup>  | 43 $\pm$ 18 <sup>a</sup> | 41 $\pm$ 18 <sup>a</sup> | 39 $\pm$ 18 <sup>a</sup> | 36 $\pm$ 17 <sup>a</sup> | 33 $\pm$ 17 <sup>a</sup> | 31 $\pm$ 17 <sup>a</sup> | 30 $\pm$ 17 <sup>a</sup> | 27 $\pm$ 16 <sup>b</sup>  |
| ER           | 92 $\pm$ 10 <sup>a</sup> | 80 $\pm$ 17 <sup>a</sup> | 69 $\pm$ 21 <sup>ab</sup> | 66 $\pm$ 20 <sup>a</sup> | 64 $\pm$ 21 <sup>a</sup> | 62 $\pm$ 21 <sup>a</sup> | 60 $\pm$ 23 <sup>a</sup> | 57 $\pm$ 22 <sup>a</sup> | 55 $\pm$ 20 <sup>a</sup> | 52 $\pm$ 21 <sup>a</sup> | 48 $\pm$ 19 <sup>ab</sup> |
| EA           | 92 $\pm$ 6 <sup>a</sup>  | 77 $\pm$ 12 <sup>a</sup> | 61 $\pm$ 17 <sup>ab</sup> | 50 $\pm$ 20 <sup>a</sup> | 46 $\pm$ 20 <sup>a</sup> | 40 $\pm$ 20 <sup>a</sup> | 36 $\pm$ 21 <sup>a</sup> | 33 $\pm$ 20 <sup>a</sup> | 32 $\pm$ 20 <sup>a</sup> | 30 $\pm$ 19 <sup>a</sup> | 29 $\pm$ 19 <sup>ab</sup> |
| ER+EA        | 89 $\pm$ 12 <sup>a</sup> | 79 $\pm$ 13 <sup>a</sup> | 70 $\pm$ 9 <sup>a</sup>   | 65 $\pm$ 12 <sup>a</sup> | 61 $\pm$ 14 <sup>a</sup> | 58 $\pm$ 13 <sup>a</sup> | 57 $\pm$ 14 <sup>a</sup> | 55 $\pm$ 13 <sup>a</sup> | 53 $\pm$ 14 <sup>a</sup> | 52 $\pm$ 15 <sup>a</sup> | 50 $\pm$ 16 <sup>a</sup>  |

*Survival to megalopa*

In experiments 1, 2 and 3 there was no significant difference in the number of Z5 larvae that survived metamorphosis to megalopa. Similarly, there were no significant differences in the number of Z1 larvae that survived to megalopa in the various treatments (Table 3).

In experiment 4, significantly more Z1 larvae survived to megalopa in treatment ER ( $p = 0.03$ ) and treatment ER+EA ( $p = 0.02$ ) than in the control. No significant differences in survival of Z1 or Z5 to megalopa were recorded between treatments ER and EA or EA and the control. A significantly higher proportion of Z5 larvae survived metamorphosis to megalopa in treatment ER+EA in comparison to the control ( $p = 0.0006$ ).

**Table 3.** Average percent survival to megalopa  $\pm$  standard deviation of *Scylla serrata* larvae fed different enrichment diets. M/Z1= % survival of zoea 1 to megalopa; M/Z5 = % metamorphosis of zoea 5 to megalopa; Control = no enrichment; ALG = *Artemia* enriched with *Tetraselmis*; SS = *Artemia* enriched with Super Selco®; DHA = *Artemia* enriched with DHA Selco®; ZERO = *Artemia* enriched with 0% (n-3) HUFA; THIRTY = *Artemia* enriched with 30% (n-3) HUFA; FIFTY = *Artemia* enriched with 50% (n-3) HUFA; ER = only rotifers enriched with Super Selco®; EA = only *Artemia* enriched with Super Selco®; ER+EA = both rotifers and *Artemia* enriched with Super Selco®. Values in the same column (for each experiment) showing the same superscript are not significantly different. ( $p \leq 0.05$ ).

| Treatment    | M/Z1                      | M/Z5                      | Replicates (n) |
|--------------|---------------------------|---------------------------|----------------|
| Experiment 1 |                           |                           |                |
| Control      | 22 $\pm$ 14 <sup>a</sup>  | 30 $\pm$ 17 <sup>a</sup>  | 6              |
| ALG          | 37 $\pm$ 22 <sup>a</sup>  | 52 $\pm$ 27 <sup>a</sup>  | 5              |
| SS           | 19 $\pm$ 13 <sup>a</sup>  | 27 $\pm$ 15 <sup>a</sup>  | 6              |
| DHA          | 26 $\pm$ 15 <sup>a</sup>  | 36 $\pm$ 18 <sup>a</sup>  | 6              |
| Experiment 2 |                           |                           |                |
| Control      | 40 $\pm$ 18 <sup>a</sup>  | 57 $\pm$ 23 <sup>a</sup>  | 9              |
| ALG          | 22 $\pm$ 14 <sup>a</sup>  | 32 $\pm$ 28 <sup>a</sup>  | 9              |
| ZERO         | 40 $\pm$ 18 <sup>a</sup>  | 29 $\pm$ 19 <sup>a</sup>  | 8              |
| DHA          | 21 $\pm$ 19 <sup>a</sup>  | 56 $\pm$ 20 <sup>a</sup>  | 9              |
| Experiment 3 |                           |                           |                |
| Control      | 37 $\pm$ 20 <sup>a</sup>  | 55 $\pm$ 28 <sup>a</sup>  | 6              |
| ZERO         | 39 $\pm$ 21 <sup>a</sup>  | 63 $\pm$ 27 <sup>a</sup>  | 8              |
| THIRTY       | 20 $\pm$ 10 <sup>a</sup>  | 40 $\pm$ 17 <sup>a</sup>  | 6              |
| FIFTY        | 24 $\pm$ 7 <sup>a</sup>   | 49 $\pm$ 17 <sup>a</sup>  | 6              |
| Experiment 4 |                           |                           |                |
| Control      | 12 $\pm$ 8 <sup>c</sup>   | 35 $\pm$ 14 <sup>b</sup>  | 6              |
| ER           | 35 $\pm$ 12 <sup>ab</sup> | 59 $\pm$ 21 <sup>ab</sup> | 6              |
| EA           | 21 $\pm$ 17 <sup>bc</sup> | 60 $\pm$ 19 <sup>ab</sup> | 6              |
| ER+EA        | 41 $\pm$ 17 <sup>a</sup>  | 75 $\pm$ 12 <sup>a</sup>  | 6              |

*Fatty acid analysis*

Rotifers enriched with Super Selco® contained double the amount of FAME than unenriched rotifers. Higher levels of EPA, DHA, ARA and total (n-3) and (n-6) HUFA were also recorded in the enriched rotifers. However the ratios of DHA/EPA and ARA/EPA were lower due to the high EPA levels in the enriched rotifers (Table 4).

In comparison to the unenriched *Artemia* nauplii, lower levels of gross FAME including EPA, ARA, total (n-3) HUFA and total (n-6) HUFA were recorded for *Artemia* enriched with *Tetraselmis*. Total FAME was increased in *Artemia* enriched with the 0% ICES emulsion, but levels of (n-3) and (n-6) fatty acids were lower. DHA was detected only at trace levels in both unenriched *Artemia* and *Artemia* enriched with *Tetraselmis*. Levels of EPA, DHA and sum (n-3) HUFA were substantially higher in the *Artemia* enriched with Super Selco®, DHA Selco®, 30% and 50% ICES emulsions than for the unenriched *Artemia* nauplii. Higher levels of total FAME, ARA and sum (n-6) HUFA were also recorded in the enriched *Artemia*. The DHA/EPA ratio was also increased by enriching with emulsions and was particularly high for *Artemia* enriched with DHA Selco®.

In both experiments 1 and 4, a substantial increase in total FAME level was recorded for Z5 which had been fed live food enriched with HUFA emulsions compared to newly hatched Z1: Z5 in the treatment groups contained higher levels of EPA, total (n-3) HUFA and total (n-6) HUFA. There was a relatively small increase in DHA levels recorded for the Z5 larvae in the treatment groups of both experiments.

In experiment 1, Z5 larvae in treatments SS and DHA contained higher total FAME levels (approximately double) than Z5 larvae in the control and treatment ALG. EPA level was more than double, DHA was increased more than sixty times, but ARA levels were relatively unchanged. Total (n-3) HUFA was tripled and total (n-6) HUFA was more than doubled. The DHA/EPA ratio was increased approximately fourfold but the ARA/EPA ratio decreased to approximately half that of larvae in the control.

In experiment 4, Z5 larvae in treatments ER, EA and ER+EA contained total FAME levels more than double those recorded for the control group. EPA was more than doubled and approximately five times the amount of DHA was recorded. Total (n-3) HUFA levels were almost three times as high for larvae in the treatment groups. The level of sum (n-6) HUFA was more than 50% higher for larvae in treatment ER and double for larvae in treatments EA and ER+EA. DHA/EPA ratio was more than doubled and ARA/EPA were close to five times higher. Amongst the treatment groups, highest levels of total FAME, EPA, DHA, ARA and

sum (n-3) HUFA were recorded for Z5 larvae in treatment ER+EA. Highest sum (n-6) HUFA level was recorded for larvae in treatment EA and highest DHA/EPA and ARA/EPA ratios were recorded in treatment ER.

In experiment 4, there was a significant linear relationship between percent metamorphosis (Z5/M) of larvae in the treatments and levels of total FAME ( $R^2 = 0.93$ ;  $p = 0.035$ ), EPA ( $R^2 = 0.94$ ;  $p = 0.030$ ) and sum (n-3) HUFA ( $R^2 = 0.89$ ;  $p = 0.037$ ). The relationship between percent metamorphosis and the other FAME was not linear. The relationships between LSI at 15 DAH, zoeal survival at 15 DAH and FAME profile of the larvae were also not linear.

There was a large variation in fatty acid profile between batches of larvae hatched over an 18 month period. Higher levels of total FAME, EPA, DHA, sum (n-3) and sum (n-6) were recorded for Z1 larvae in experiment 1 than in experiment 4.

**Table 4.** Fatty acid methyl ester (FAME). Profiles of live food and *Scylla serrata* larvae at 15 days after hatch. EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; ARA = arachidonic acid; Control = no enrichment; ALG = *Artemia* enriched with *Tetraselmis*; SS = *Artemia* enriched with Super Selco®; DHA = *Artemia* enriched with DHA Selco®; ZERO = *Artemia* enriched with 0% (n-3) HUFA; THIRTY = *Artemia* enriched with 30% (n-3) HUFA; FIFTY = *Artemia* enriched with 50% (n-3) HUFA; ER = only rotifers enriched with Super Selco®; EA = only *Artemia* enriched with Super Selco®; ER+EA = both rotifers and *Artemia* enriched with Super Selco®; Z1 = newly hatched first zoeae; Z5 = fifth stage zoeae; \*  $\geq 20:3(n-3)$ ; \*\*  $\geq 18:2(n-6)$ .

|                                                                   | Fatty Acid levels (mg/g) |      |      |     |                     |                      |         |         |
|-------------------------------------------------------------------|--------------------------|------|------|-----|---------------------|----------------------|---------|---------|
|                                                                   | Total FAME               | EPA  | DHA  | ARA | $\Sigma(n-3)$ HUFA* | $\Sigma(n-6)$ HUFA** | DHA/EPA | ARA/EPA |
| <b>Live food</b>                                                  |                          |      |      |     |                     |                      |         |         |
| Unenriched                                                        |                          |      |      |     |                     |                      |         |         |
| Rots.                                                             | 48.6                     | 3.4  | 2.6  | 0.9 | 9.2                 | 6.6                  | 0.76    | 0.26    |
| SS enriched                                                       |                          |      |      |     |                     |                      |         |         |
| Rots.                                                             | 96.7                     | 9.4  | 4.1  | 1.2 | 18.4                | 11.7                 | 0.43    | 0.13    |
| <i>Artemia</i> nauplii                                            | 180.8                    | 5.5  | 0.1  | 1.9 | 6.5                 | 13.7                 | 0.01    | 0.34    |
| ALG <i>Artemia</i>                                                | 80                       | 1.6  | 0.2  | 0.5 | 3.1                 | 6.2                  | 0.13    | 0.31    |
| ZERO <i>Artemia</i>                                               | 191                      | 1.9  | 0.1  | 0.2 | 2.2                 | 5.3                  | 0.05    | 0.12    |
| THIRTY                                                            |                          |      |      |     |                     |                      |         |         |
| <i>Artemia</i>                                                    | 191.2                    | 29.6 | 11.2 | 2.5 | 44.1                | 14.8                 | 0.37    | 0.08    |
| FIFTY <i>Artemia</i>                                              | 216.3                    | 33.7 | 16   | 2.4 | 54.0                | 16.1                 | 0.47    | 0.07    |
| SS <i>Artemia</i>                                                 | 206.4                    | 29.3 | 15.6 | 3.1 | 50.7                | 16.9                 | 0.53    | 0.33    |
| DHA <i>Artemia</i>                                                | 176.0                    | 9.3  | 14.4 | 2.3 | 26.6                | 14.7                 | 1.55    | 0.25    |
| <b>Larvae</b>                                                     |                          |      |      |     |                     |                      |         |         |
| Experiment 1                                                      |                          |      |      |     |                     |                      |         |         |
| Z1                                                                | 49.8                     | 8.2  | 5.1  | 1.8 | 17.7                | 2.8                  | 0.62    | 0.22    |
| Z5 Control                                                        | 48.7                     | 5.7  | 0.6  | 1.8 | 7.7                 | 4.5                  | 0.11    | 0.32    |
| Z5 ALG                                                            | 40.7                     | 5.9  | 0.8  | 1.9 | 7.7                 | 3.8                  | 0.12    | 0.32    |
| Z5 SS                                                             | 126.3                    | 15.9 | 6.7  | 2.3 | 26.0                | 10.5                 | 0.42    | 0.15    |
| Z5 DHA                                                            | 109.6                    | 13.3 | 8.5  | 2.1 | 21.2                | 9.4                  | 0.63    | 0.16    |
| Experiment 4                                                      |                          |      |      |     |                     |                      |         |         |
| Z1                                                                | 35.2                     | 4.3  | 4.4  | 1.5 | 9.3                 | 2.4                  | 1.01    | 0.34    |
| Z5 Control                                                        | 51.3                     | 5.2  | 0.9  | 1.7 | 7.5                 | 4.6                  | 0.17    | 0.32    |
| Z5 ER                                                             | 101.4                    | 12.0 | 5.0  | 1.8 | 19.3                | 7.9                  | 0.41    | 0.15    |
| Z5 EA                                                             | 116.6                    | 12.7 | 4.8  | 1.8 | 19.9                | 12.7                 | 0.38    | 0.14    |
| Z5 ER+EA                                                          | 124.6                    | 14.0 | 5.3  | 2.0 | 21.9                | 10.5                 | 0.38    | 0.14    |
| Newly hatched Z1 larvae averaged over 16 batches during 18 months |                          |      |      |     |                     |                      |         |         |
| Average                                                           | 47.2                     | 6.0  | 5.9  | 1.4 | 13.0                | 2.9                  | 0.97    | 0.26    |
| Std. deviation                                                    | 23.5                     | 3.0  | 3.2  | 0.5 | 5.8                 | 1.9                  | 0.36    | 0.10    |

## Discussion

Feeding HUFA enriched *Artemia* to larvae in the treatment groups of all the experiments resulted in elevated HUFA levels in the larvae (Table 4). There was, however, no corresponding (significant) improvement in development, survival or metamorphosis as a result (Tables 1, 2 and 3). Survival through metamorphosis was not significantly compromised for larvae in treatments ALG and ZERO in experiments 1 and 3 respectively, in which the larvae were essentially starved of HUFA. Similar results were reported by Mann *et al.* (2001) who recorded no significant improvement in performance of *S. serrata* in Australia, despite similarly feeding the larvae with Selco® enriched *Artemia*. Kobayashi *et al.* (2000) also recorded no improvement in survival of *S. tranquebarica* larvae fed on HUFA enriched *Artemia* in comparison to larvae fed on unenriched *Artemia*. The results of the experiments therefore support Mann *et al.*'s (2001) suggestion that boosting *Artemia* with HUFA has no significant effect on the performance of *Scylla serrata* larvae.

However, when both rotifers and *Artemia* were enriched with Super Selco® (treatment ER+EA in experiment 4), development, survival and metamorphosis of the larvae were all significantly improved (Tables 1, 2 and 3). When only the rotifers were enriched in experiment 4, significant improvements in early development (Table 1), early zoeal survival (Table 2) and survival of Z1 and Z5 (Table 2 and Fig. 2) to megalopa (Table 3) were recorded. Hamasaki *et al.* (2002a, b) and Suprayudi *et al.* (2002b) recorded similar improvements in metamorphosis for *S. serrata* larvae in Japan as a result of feeding HUFA enriched rotifers and unenriched *Artemia* nauplii. In recent experiments in Vietnam, significantly faster development was recorded for *S. paramamosain* up to 15 DAH as a result of feeding rotifers enriched with HUFAs for the first 6 DAH (Vandendriessche 2003). This indicates a requirement for HUFA enrichment early in the rearing period of *S. serrata* larvae. The lack of significant improvements in growth and survival of larvae recorded by Mann *et al.* (2001) may have been due to the fact that only the *Artemia* were enriched in their study.

*S. serrata* embryos use lipids and fatty acids in the yolk as a source of energy and tissue building respectively (Cheng and Li 2001). This is reflected by a decrease in the levels of certain fatty acids during embryonic development (Churchill 2003) and the larvae therefore have a very small reserve of fatty acids at hatch (Cheng and Li 2001). There is, however, a significant increase in the proportion of (n-6) and (n-3) HUFAs (particularly EPA) during embryonic development (Churchill 2003), suggesting that these fatty acids may be set aside by the embryo and implying a requirement of these fatty acids by the larvae for early

development. Boosting FAME levels (including HUFAs) in the rotifers fed at hatch may have given the larvae a significant advantage which was carried through the rearing process and culminated in improved survival through metamorphosis, particularly in treatment ER+EA where the HUFA supply was uninterrupted. This phenomenon has been recorded for other crustacean larvae (including crabs (Sulkin 1978)) and the effects of early nutrition often manifest during the latter stages of development and particularly at metamorphosis to the post larval stages (megalopa in crabs) (Sulkin 1978; Harvey 1996; Jeffs *et al.* 1999; Ribeiro and Jones 2000).

Regression analysis revealed significant linear relationships between successful metamorphosis (M/Z5) and levels of total FAME, EPA and sum (n-3) HUFA in the Z5 in experiment 4. This suggests that high levels of these fatty acids were required by the larvae for successful metamorphosis. However, high levels of these fatty acids were also recorded for larvae in the SS and DHA treatments of experiment 1 without a corresponding improvement in survival. High HUFA levels in crustacean larvae do not necessarily result in improved performance (González-Félix *et al.* 2002) and the performance of *S. serrata* larvae can even be compromised when HUFA is supplied at excessive levels (Suprayudi *et al.* 2002b). It is therefore suggested that it was not only the absolute levels of HUFA in the feed that were important but also the timing that the HUFAs were delivered to the larvae. The stage of development at which particular fatty acids are delivered to the larvae is important as the fatty acid composition of crustacean larvae changes with growth (Wouters *et al.* 1997). Fatty acid requirements of the larvae therefore change accordingly (Rees *et al.* 1994; Coutteau *et al.* 1997; Roustaian *et al.* 1999). It is possible that the HUFAs contained in Super Selco® are only essential for *S. serrata* during the early stages of development and that later stage larvae require different HUFA enrichment. Such a change in HUFA requirements with development has been recorded for *S. paramamosain* larvae in Vietnam (Vandendriessche 2003). High DHA levels and DHA/EPA ratio of the Z5 larvae in treatments SS and DHA (Experiment 1) did not significantly enhance larval performance. The relationship between survival or growth and DHA levels in the Z5 larvae was not linear in experiment 4 and DHA levels for Z5 in treatment ER+EA were not much higher than those in treatments ER or EA. The performance of larvae thus cannot be linked to DHA levels in the diet. This contrasts with evidence that DHA is important for the larvae of several other carnivorous crustacean species including the southern rock lobster *Jasus edwardsii* (Nelson *et al.* 2003) spiny lobster *Panulirus japonicus* (Kanazawa and Koshio 1993), the blue crab *Callinectes sapidus* (Levine and Sulkin 1984) and *S. paramamosain* in Vietnam (Vandendriessche 2003). It is possible that a connection between

DHA levels and larval performance in our study could have been obscured by the fact that FAME analysis could not be performed on the larvae after the rotifer feeding period. The FAME profile of the Z5 larvae in Table 4 is more indicative of the effects of feeding enriched *Artemia* during the latter stages of larval development than the effects of feeding enriched rotifers during early development. It is thus difficult to speculate which of the fatty acids in the enriched rotifers and *Artemia* were essential. In addition, there are nutrients other than fatty acids in Super Selco® including vitamins C and E. Enrichment of live food with these nutrients has resulted in improved performance of cultured larvae (Merchie *et al.* 1997; Neelkamal 1998; Kolkovski *et al.* 2000). It is therefore possible that an increase in either of these vitamins could have resulted in the differences recorded in experiment 4.

The results have shown that larval performance of South African *Scylla serrata* is enhanced when both rotifers and *Artemia* are enriched with Super Selco®. However, in this suite of experiments certain anomalous results were recorded. This is not uncommon and raises the question about the cause of inconsistent results in crab larval nutrition studies. For example, highest total FAME was recorded for larvae in the ER+EA treatment and the linear relationship between successful metamorphosis and total FAME in Z5 larvae in experiment 4 implies that high levels are required by the larvae to complete metamorphosis. This could indicate a minimum level of FAME to fulfil the energetic requirements of metamorphosing larvae (Mead *et al.* 1986) and that boosting total FAME via both the rotifers and *Artemia* facilitated this increase. However the high total FAME level recorded for Z5 larvae in treatment SS in experiment 1 did not result in significantly improved performance and there was no significant correlation between FAME level in the Z5 larvae and metamorphosis in this experiment. One of the suggested causes for unpredictable results in mud crab larval nutrition studies has been variability in quality between different batches of larvae (Mann *et al.* 1999a; Zeng and Li 1999; Millamena and Bangcaya 2001; Djunaidah *et al.* 2003). In this study, highly variable FAME profiles were recorded for South African *S. serrata* larvae sampled from 16 different batches over an 18 month period (Table 4). Higher total FAME, EPA and DHA were recorded for Z1 in experiment 1 compared to experiment 4. Z1 in experiment 4 had lower amounts of all fatty acids than the average of 16 batches of larvae produced over 18 months (Table 4). This may indicate that the larvae had a higher requirement for HUFA at hatch and may explain why enrichment of the live food (particularly the rotifers) in experiment 4 had a significant effect on the larvae whereas enriching the live food in experiment 1 did not. High variability in the fatty acid content of eggs produced by wild-caught broodstock is not uncommon (Peleteiro *et al.* 1995) and this was also recorded for *S. serrata* in South Africa

(Churchill 2003). In addition, although broodstock crabs were fed the same diet during the 18 month period, they each spent different times in captivity before extruding their eggs (Davis *et al.* 2003a). The nutritional status of female mud crabs affects that of the eggs and the Z1 larvae (Li *et al.* 1999; Djunaidah *et al.* 2003) which may explain the variability in FAME profiles recorded. The HUFA content of *S. serrata* larvae (particularly EPA and total (n-3) HUFA) has been linked to larval quality (Churchill 2003), and larvae containing different fatty acid profiles at hatch may require different levels or ratios of certain EFAs in the diet. This would suggest that in future, studies on the fatty acid requirements of *Scylla* larvae, the FAME profile of the eggs needs to be established prior to making any comparisons between treatments and that comparisons between larval rearing treatments should only be made for batches with similar egg FAME profiles. Perhaps by following this routine we may expect more consistent results in *S. serrata* larval nutrition studies in future.

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# Chapter 8

## General discussion

### **Understanding the difficulties associated with larval rearing**

In order to appreciate the challenges involved with the captive rearing of *Scylla* larvae, it is necessary to understand the nature of marine larvae in general and the environment to which they have evolved. Many commercially important marine species have evolved “r” selected life history strategies for which large numbers of eggs are produced. Because there is a finite amount of energy that the breeding female can invest in each brood, the eggs of fecund species are small and usually contain correspondingly small amounts of yolk – often insufficient to support complete development of the definitive phenotype (first juvenile stage). The balance of the nutrition required to complete development therefore has to be acquired from an exogenous source. This is the temporary larval stage whose function is “an external nutrient acquiring device” (Balon 1986). For benthic species (such as *Scylla*), there are a host of advantages to producing large numbers of small larvae with extended planktonic development. It facilitates gene exchange between populations which maintains genetic variability; it allows for colonization of areas remote from the main population; it overcomes the possibility of extinction due to unpredictable habitat disturbance; ensures a certain percent survival to adulthood when the juveniles are vulnerable to predation; reduces competition between the larvae and ensures that the adults occupy a separate niche from the larvae (Sastry 1983; Balon 1986). There are however certain disadvantages. The smaller the yolk, the sooner the larvae hatch and the longer they need to spend in the (more vulnerable) larval state. Smaller larvae are lower in the food chain as they are restricted to smaller food particles and are preyed upon by a wider variety of larger planktonic predators. Pelagic larvae can also be transported to unfavorable areas by water currents; food conditions may vary unpredictably in space and time, and juvenile recruitment may be uncertain (Sastry 1983). Although the larvae are

vulnerable, poor survival is compensated for by the large numbers produced (Balon 1986). The early life history strategy of many marine species can therefore be described as pre-adapted to high mortality - survival of fish eggs and larvae in the wild has been estimated at less than 5% (Barnabé 1990). Whereas this life history strategy serves marine species in the wild, a small, underdeveloped larva with an extended development time makes rearing in captivity extremely difficult. This is further aggravated by the fact that only a small proportion of the larvae produced (especially by more fecund species) can be accommodated in the hatchery and high survival is required in order to make captive rearing viable (Wickins and Lee 2002).

Crustacean larval development occurs within a narrow range of environmental parameters (Sastry 1983). The conditions that planktonic marine larvae encounter in the wild are impossible to recreate in the laboratory. The ocean acts as a buffer into which harmful chemicals are quickly diluted and changes in temperature, salinity or pH occur extremely slowly. Larvae are able to maintain a minimum distance from one another and dead or diseased larvae drop out of the surface layers and do not pollute the water. The larvae are also able to regulate their position in the water column, selecting ideal conditions of light, salinity, turbulence (Forward *et al.* 1984) and food availability (Malkiel *et al.* 1999). The distribution of plankton in the ocean is not homogenous (Benfield and Downer 2001) as oceanographic phenomena tend to concentrate plankton in “fronts” (Clark *et al.* 2001) or “patches” (Natunewicz *et al.* 2001). Crab larvae that are incorporated into these fronts are vulnerable to predation from larger zooplankton, but in turn have access to an abundance of smaller plankton as food.

In the hatchery, predators are excluded from the rearing vessels but the larvae are crowded together into small volumes, at artificially high densities, increasing interactions and the risk of disease and cannibalism. Smaller volumes of water are subject to changes in physical and chemical parameters. Although live food is always made available, the species provided are selected as much for their ease of culture as for their suitability as feed and usually bear little resemblance to the natural food of the larvae in the wild. Cultured live food organisms are often stressed from the rigors of mass culture and processing, carry bacteria and other contaminants and sometimes lack essential nutrients (Støttrup and McEvoy 2003).

Mud crabs invest a certain amount of reproductive effort in their offspring. The females both care for the nauplius stage while it develops within the egg (Sastry 1983) and migrate offshore to release the zoeae in conditions more favorable to their development (Hill 1994). However, females extrude millions of very small eggs in each of the few spawning events that occur during their relatively short lives (3-4 years (Hill 1975)) (Chapter 4) which hatch into

very small zoeae that have an extended developmental period (Brick 1974). *Scylla serrata* tend towards the altricial end of the altricial-precocial life-history continuum (Stearns 1976) and can be said to have evolved a relatively “r” selected life history strategy. This life history strategy has resulted in *Scylla* being widely distributed within a relatively short evolutionary timescale (Fratini and Vannini 2002). Mitochondrial DNA studies have revealed that *S. serrata* colonized the entire Indian Ocean by means of a single expansion event from the West Pacific region during the Pleistocene (approximately 1 million years ago) (Gopurenko *et al.* 1999). The fact that *S. serrata* females migrate further offshore than the other *Scylla* species to release the larvae (Ng 1998; Emilia Qunitio, Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan, Iloilo, The Philippines, pers. comm.), could explain why they are more widely distributed. Mud crabs are also able to colonize suitable environments relatively quickly (Forbes and Hay 1988) and can thus probably withstand local, isolated extinction events (Chapter 3). This has resulted in mudcrabs being relatively common animals in a variety of habitats (Le Vay 2001).

However, this life history strategy means that newly hatched *Scylla* larvae are small (less than 0.3 mm lateral spine width) (Chapter 4), underdeveloped (Zeng and Li 1999), have very little yolk reserve (Cheng and Li 2001) and need to feed soon after hatch (Li *et al.* 1998; Lumasag and Qunitio 1998; Li *et al.* 1999; Djunaidah *et al.* 2003). They are inefficient predators (Heasman and Fielder 1983) and therefore require high densities of live food (Zeng 1998). But they are extremely susceptible to disease (Mann 2001) and thus require meticulously hygienic conditions (Blackshaw 2001). They are sensitive to subtle changes in their physical environment (Hill 1974) and require stable, high quality seawater (Chapter 2). They are also highly cannibalistic (Dat 1999; Qunitio *et al.* 2001; Suprayudi *et al.* 2002a) and undergo two energetically demanding and stressful metamorphoses before reaching the juvenile stage (Hamasaki *et al.* 2002b). The larvae are thus extremely difficult to rear *en masse* and establishing hatchery technology has proven to be more challenging than other commercially important decapod crustaceans such as the penaeid shrimp, crab species such as the Chinese mitten crab (*Eriocheir sinensis*) (Naihong *et al.* 1999) and other portunids such as the blue crab (*Portunus trituberculatus*) (Cowan 1984; David Mann, Bribie Island Aquaculture Research Center, Brisbane, Australia, pers. comm.). However, mud crab larvae are not as difficult to rear as those of the palinurid spiny lobsters (mostly *Panulirus* spp.) which produce both a sensitive phyllosoma and a non-feeding puerulus larval stage, that both take a long time to develop (197-365 days) (Kittika 1994; Wickins and Lee 2002). Small numbers of mud crab larvae can be reared to megalopa using relatively simple technology as was demonstrated by

pioneering researchers (Ong 1964; Du Plessis 1971). This has often led to initial excitement when mud crab larval rearing research projects begin. However, it is the breakthrough to predictable, repeatable, high survival which has escaped researchers thus far. Despite over twenty years of intensive research in Japan and a decade of research in South East Asia, seed production is still not practiced commercially (Chapter 2). Predictions during the fact finding mission in 1997 and the International Forum on the Culture of Portunid Crabs held in Boracay, Philippines in December 1998 that the technology for mass seed production was imminent, have still not materialized. By the start of the larval rearing project in 1999, it was realized that the technology was not suitable for transfer and that hatchery technology for South African *Scylla serrata* would therefore have to be developed from the beginning.

### Genetics

The genetic study revealed that mudcrabs along the east coast of South Africa belong to a single homogeneous group with limited geographic structuring, indicating that juveniles probably recruit from a common regional pool. Although this needs to be verified by larger studies using more sophisticated techniques such as validated polymorphic microsatellite DNA markers (Sekino *et al.* 2003), this has several implications for the future of mudcrab aquaculture in the region.

Firstly, it is probable that this genetic homogeneity extends at least into southern Mozambique. Gopurenko *et al.* (1999) found that the South African mudcrab population (sampled in Durban) consisted of a single mitochondrial haplotype found in no other population. However, they did not sample crabs from Mozambique and our results support gene flow over the length of the South African Indian Ocean coast, which would probably extend into Mozambique. Although this needs to be verified as described above, the indication is that possible future collaborations in mudcrab aquaculture between South Africa and Mozambique would not necessarily be restricted by large genetic differences between *Scylla serrata* in the region.

Secondly, translocation of mudcrabs between estuaries is unlikely to have serious genetic consequences as recruitment of megalopae into the estuaries would seem to be random and the RAPDs data support the idea that estuary populations are not genetically distinct. It is important that sufficient care be taken to avoid genetic drift and genetic impoverishment. Genetic heterogeneity can be improved by ensuring a balanced parental sex ratio and a high turnover of broodstock (Sekino *et al.* 2003). The spawning biology of mud crab broodstock

goes some way to ensuring this. An extended spawning embrace (up to one week) is followed by implantation of spermatophores, improving the chances of a 1:1 sex ratio. Although the ovaries of wild caught females can re-mature in captivity, the eggs of only one spawning are generally used before the females are returned to the wild. The risk of compromising the genetic integrity of wild populations through the inter-estuarine transfer of juveniles for ranching or stock enhancement could therefore be reduced by following this practice. Once hatchery technology is established, the potential for stock enhancement of mud crabs in South Africa is good as crabs are prevented from moving between estuaries by the high energy coastline (Perrine 1979; Hyland 1984; Le Vay 2001).

Thirdly, that technology developed in hatcheries using broodstock caught in Kwazulu Natal, for example, can probably be applied to mudcrab in the entire region.

Fourthly, the larvae do not necessarily recruit back into the estuary of parental origin. In areas of Taiwan where mud crabs are farmed, recruitment of juveniles is unusually high (Jin-Hua Cheng, Tungkang Marine Laboratory, Taiwan Fisheries Research Institute, Tungkang, Pingtung, Taiwan, ROC, pers. comm.). Decapod crustacean larvae generally remain in the hatching area of adults (Sastry 1983). The recruitment has been attributed to females (which had escaped from production ponds in estuaries) releasing larvae in the area which then recruited back into the estuaries. This suggested the possibility that releasing large numbers of newly hatched larvae in the proximity of an estuary could increase juvenile recruitment in the region which could be used as seed, delaying the need for hatchery produced juveniles. This may be possible in Taiwan where species (*S. paramamosain* and *S. olivacea*) are farmed which release their larvae closer inshore than *S. serrata* (Ng 1998; Emilia Quintio pers. comm.). However, our data showed that this approach cannot be applied in South Africa, further emphasising the need for hatchery produced seed.

### **Broodstock**

Capturing broodstock consumed a great deal of time and labour. The Umlalazi estuary is a proclaimed estuarine sanctuary - special permission to catch broodstock was granted for research purposes- and therefore has higher crab densities than other estuaries in the country (Robertson 1996). Despite this, the CPUE for mature females was low and variable, ranging from 0.05 crabs per trap hour in July and August to 0.16 in March and June. Under extreme weather conditions, fishing was not possible. Sufficient crabs were caught to provide eggs for larval rearing research, but future projects will require a more regular supply of broodstock.

Pond reared crabs produce good quality eggs (Millamena and Quinitio 2000) and the life cycle has been closed in captivity (Quinitio *et al.* 2001b). This offers the opportunity for developing a domesticated broodstock. This could not only ensure a predictable supply of eggs, but offers the culturist a much greater degree of control over all aspects of reproduction (Chamberlain 1985).

Poor egg quality can be a significant problem for the larviculture of any new species (Bromage 1995). Of the many factors that influence egg quality, the two factors which can be controlled by the culturist are the extent to which the female brood animals are exposed to stress and their nutritional status (Bromage 1995). The nutritional status of the crabs caught in the Umlalazi estuary was unknown. Although all broodstock received the same diet, females spawned after different times in captivity, which (as discussed in Chapter 7) may explain the high degree of variation in FAME profile between larvae from different batches. The importance of early nutrition to South African *S. serrata* larvae was highlighted in chapter 8 where enriching both rotifers and *Artemia* with (n-3) HUFA significantly enhanced larval performance compared with the enrichment of *Artemia* alone. When the nutrition of newly hatched larvae was compromised by a lack of appropriate live food in chapters 6 and 7, the larvae performed poorly throughout the remaining developmental period. This was despite the introduction of suitable feeding later in the rearing period. There is a direct link between broodstock nutrition and larval quality (Bromage 1995; Cavalli 2000) and improving the nutrition of mud crab broodstock has resulted in enhanced larval performance (Millamena and Quinitio 2000). Highly variable results for mud crab larval rearing experiments recorded during this project and elsewhere have been attributed to variability in larval quality at hatch (Mann *et al.* 1999; Zeng and Li 1999; Millamena and Bangcaya 2001). Although Churchill (2003) found no correlation between FAME profile and the ability of larvae to withstand stress, a domesticated broodstock fed an appropriately formulated diet could help standardise the nutritional status of the hatched larvae, introduce more predictability to larval rearing and improve larval performance throughout the rearing period. It should be noted that if hatchery produced seed are to be used for restocking or ranching purposes, wild caught broodstock will still be required in order to maintain genetic variation, the loss of which can lead to poor survival and growth of the animals in the wild (Allendorf and Phelps 1980).

Peak spawning in the laboratory for *S. serrata* caught in the Umlalazi estuary were slightly different from those recorded in the wild for the same population (Robertson and Kruger 1994). The suggestion that spawning was affected by captive conditions was supported by the pattern of coordinated spawning (Chapter 3, Figure 7). Manipulating spawning through

environmental change could provide a useful tool for broodstock management, particularly for pond reared animals. Practically, however, coordinated spawning was often problematic. When several crabs spawned simultaneously, there was an overabundance of larvae and as comparatively few could be accommodated in the hatchery, the majority were wasted. This was separated by periods where no females spawned and larval rearing experiments were thus delayed. On the other hand, having several batches of eggs available simultaneously, allowed for the selection of the “best” batch for further rearing experiments. Batches of eggs were initially chosen through visual observation. Females with dark orange eggs, strongly attached to the pleopods were selected. However, when the quality of the larvae hatching from these batches was tested, no analytical data could be found to support these choices (Churchill 2003). It is unknown to what extent spawning in captivity is affected by the animal’s history in the wild. A domesticated broodstock would provide more control over spawning and larval quality.

In South Africa, indications were that producing a domesticated *Scylla serrata* broodstock would not be difficult: Juvenile crabs caught from the wild, from drained shrimp ponds at APF and reared from egg during early larval rearing trials were stocked into a fertilised pond at MPF where they were fed waste prawn shells from the farm’s processing plant and commercial shrimp grow-out feed. Several females spawned in this pond and produced healthy batches of eggs. This activity was terminated when (understandable) concerns were raised about the possible threat to the HACCP status of MPF.

### **A practical approach to establishing larval rearing techniques**

Zootechnics, disease and nutrition are the three main areas of research which have led to commercial hatchery production for marine fish and crustacean larvae (Sorgeloos and Léger 1992). The three aspects are interconnected to some extent and developing hatchery technology for “new” species is not possible unless all three are addressed. There has been a great deal of progress in marine larval rearing technology since its beginnings in the 1960’s (Shelbourne 1964; Howell *et al.* 1998). Many of the more technical aspects developed in the past can be directly applied to new species. Some modification is usually required to achieve acceptable survival rates, but if mass mortality persists, more specific research becomes necessary. There is a myriad of possible causes for larval mortality which can be investigated and the choice of which aspects to tackle first will depend on the nature of the species and the available resources.

A variety of systems were developed for mass rearing mud crab larvae during the project. The most successful results were achieved when larvae were stocked at 60/L into 100 L, cylindroconical, black, fiberglass tanks, provided with upwelling seawater which was recirculated through a central biofilter. Before being used in the system, the seawater was pre-filtered down to 1  $\mu\text{m}$ , sterilised with ozone, passed through activated carbon and then recirculated through biofilters containing crushed oyster shell, nylon “wool” and activated carbon. The biofilters were inoculated and matured using a suspension of selected nitrifying bacteria acquired from the Artemia Reference Center (ARC), Ghent University, using methods described by Grommen *et al.* (2002). Water was recirculated for three days before the larvae were introduced into the rearing tanks. Recirculation was shut off before the larvae were introduced and *Chlorella* spp. was added to the water at a concentration of  $5 \times 10^4$  cells/ml. There was no recirculation for the first three days of rearing and thereafter water exchange was slowly increased to a maximum of 70% of the volume daily. Outlet screens were cleaned and changed, and uneaten food was flushed from the system daily. A 12 hour photoperiod was provided using a 1200  $\text{lm/m}^2$  light source. A variety of zootechnics were investigated in this system including larval stocking density, food density, feeding frequency, transferring larvae to clean tanks at Z3 and the application of prophylactics against disease.

At the beginning of the project, mass mortality was recorded early in the rearing process (during the first zoeal stage), but as zootechniques improved, healthy cultures were maintained for longer periods. Although acceptable survival to the megalopa stage could occasionally be achieved in individual tanks (5-10%) this was highly variable, both between tanks and between experiments and results were thus inconclusive. It was difficult to accurately determine survival during the early (Z1-Z3) stages. Disease at the Z4 stage and mass mortalities of zoea 5 larvae during metamorphosis to megalopa made analysis of the results difficult. In order to ensure scientific progress, a rearing system was designed which could provide more control over the various parameters, particularly sources of bacterial contamination. In this system, larvae were reared in 2 L containers and manually transferred to new containers containing fresh seawater and food daily (described in Chapters 5,6 and 7). Although standard deviation within the treatments of this system was still relatively high, good survival rates were achieved and results could be statistically analysed. The results presented in chapters 5, 6 and 7 were produced in this system. The system was also used to examine preferred light intensity and to investigate the role of antibiotics in larval survival. The importance of zootechnics and systems design to successful mud crab larval rearing cannot be underestimated. Recent advances in commercial mud crab larviculture in Australia have been

partly attributed to a complex system of airlifted, upwelling water which keeps the larvae away from the sides and bottom of the container (Field 2003; Graham Williams, Darwin Aquaculture Center, Department of Primary Industry and Fisheries, Channel Island, Darwin, Australia, pers. comm.). The facilities and manpower available at MPF during the project were unfortunately not sufficient to examine the problem in enough detail. Future mudcrab hatchery projects will need to do so if appropriate systems for mass culture are to be developed.

High densities of larvae, feed organisms and waste products can result in extremely high numbers of pathogenic bacteria (Lavilla-Pitogo *et al.* 2002), increasing the chance of infection. Crustaceans in general have a primitive, non adaptive immune system (Bartlett *et al.* 2002) and once pathogenic bacteria in the water have penetrated a damaged exoskeleton or are ingested with contaminated live food, the larvae die soon after and if they are not immediately removed, disease spreads quickly. Bacterial disease has been highlighted as one of the main causes of mass mortality in mud crab larviculture (Baylon and Failaman 2001, Mann *et al.* 2001) and acceptable survival rates still depend on the addition of prophylactic antibiotics or other undesirable antibacterials to the culture water (Parado-Esteba and Quintio 1998; Field 2003; Truong Trong Nghia, pers. comm.). The only conclusive results achieved during mass rearing experiments in 100 L tanks was that the addition of 5 mg/L antibiotic (Oxytetracycline) to the rearing water as a prophylactic significantly improved survival of the larvae to megalopa as opposed to the addition of formalin (20 mg/L every second day) or untreated seawater. The link between mortality and bacterial disease was confirmed in parallel small-scale experiments: Survival was significantly enhanced and the number of *Vibrio* colony forming units (CFU) sampled from the larvae was an order of magnitude lower in those treatments that received antibiotics. The efficacy of antibiotics in mudcrab larviculture is well known (e.g. Mann 2001), but bacteria can develop resistance to antibiotics and their use in aquaculture is unsustainable (Verschuere *et al.* 2000; Irianto and Austin 2002). Probiotic microorganisms can benefit crab larvae by competing with pathogenic bacteria (Nogami and Maeda 1992) and formalin can be used to reduce bacterial load (Hameed and Balasubramanian 2000). Both have been used as alternatives to antibiotics in aquaculture and the effects of both on larval performance were investigated in 2 L bottles. These experiments served only to confirm the benefits of antibiotics and to highlight the importance of understanding the microbiology associated with mudcrab larviculture. However, investigating the bacterial flora associated with marine aquaculture is not simple. The bacterial community associated with mud crab rearing is highly volatile (Mann 2001). Although plating samples on nutrient gels can be used to broadly classify communities and estimate bacterial numbers, this does not necessarily represent conditions *in vivo* as

bacteria form dynamic communities which are influenced by a large number of variables – not least the plating procedures themselves (Verschuere *et al.* 2000). More sophisticated microbiological techniques are difficult to establish and require specialised facilities and personnel, which were not available at MPF. A coordinated research effort into the microbial community associated with South African *S. serrata* larvae is urgently required.

The significance of nutrition to marine larvae is best illustrated by reiterating Balon's (1986) definition of the larval stage as “an external nutrient acquiring device”. Since the primary evolutionary role of the larval stage is to gather food, it follows that the nature of that food will have the most influence on its development. Most zootechnical and disease problems are directly related to feeding (Sorgeloos and Léger 1992). Live feed can be a major route for harmful bacteria (Rombaut 2001) and the susceptibility of larvae to diseases and stress can be reduced with improved nutrition (Mourente and Rodríguez 1997). It was thus decided to concentrate on establishing the optimal feeding requirements for the larvae and then with the backup of the ARC to investigate their nutritional requirements. In the meanwhile, zootechnical techniques were being constantly improved. Disease was combated by incessantly improving hygiene at all stages and for all aspects of the rearing process.

### **Developing an appropriate feeding regime**

Providing rotifers at first feeding resulted in significant improvements in larval performance (Chapter 5). It was assumed that the newly hatched larvae were better able to catch and consume rotifers than *Artemia* nauplii. Providing smaller strain *Artemia* nauplii ( $\pm 457 \mu\text{m}$ ) did improve larval survival, indicating that the size of the first live food was probably not as important as its other qualities such as digestibility, behaviour or nutrition. *Scylla* larvae do not need to swallow their food whole as they tear their prey apart using the maxillules (Craine 1999) and first feeding in mud crab larvae is thus not as challenging a bottleneck as it is for many other marine species (particularly fish) (Barnabé 1991). However the importance of nutrition during the early larval stages was highlighted during all the feeding experiments. Using rotifers as a first food provided an opportunity to introduce nutritional supplements to the newly hatched larvae. Adult rotifers can be enriched with essential micronutrients within a relatively short time period ( $\pm 6$  hours) (Dhert 1996). Effective enrichment of *Artemia* nauplii takes much longer ( $\pm 24$  hours) by which time the nauplii are too large to use as a first feed (Van Stappen 1996). Rotifers are thus a necessary component of the larval diet for *Scylla serrata*.

Culturing rotifers predictably at sufficiently high densities was one of the more difficult technical aspects of the project and the lack of rotifers during early rearing experiments may partly explain poor survival. Eventually a system was adapted from similar systems in Vietnam and the ARC where rotifer densities of approximately 1000/ml could be maintained and from which approximately 6 million rotifers per day could be harvested. The system was inexpensive and simple to build, but required a high labour and management input. High density rotifer cultures are unpredictable (Fulks and Main 1991; Fu *et al.* 1997; Suantika 2001) and future projects will need to develop efficient systems.

Controlling bacterial contamination of rotifers (especially after enrichment) is much more difficult than for *Artemia* as *Artemia* cysts can be sterilised before and during hatching (Van Stappen 1996). Systems were developed for rinsing rotifers with UV sterilised seawater, but it was difficult to determine whether this was sufficient. The microbial flora will need to be controlled in future production systems and there is evidence that this can be achieved by using a recirculating system of ozone treatment combined with the inoculation of the biological filter with selected probiotic bacteria (Gatesoupe 1991; Rombaut *et al.* 2001).

Exchanging water in the larval rearing tanks during the rotifer feeding period was problematic as the 60 µm screens required to retain them blocked quickly despite aeration around the base. If larger screens were used, rotifers had to be provided regularly in order to maintain the required density (30-40 rotifers/ml). This put additional burden on the production system, but ensured that the larvae had constant access to fresh, good quality rotifers.

Although rotifers were a more suitable first food, the larvae were able to consume *Artemia* nauplii from hatch and benefited from their early addition to the diet. Larvae were also reared with good results (up to 40% to megalopa) on a diet of *Artemia* nauplii alone (Chapter 7). If required, the dependence on rotifers can be reduced by supplementing the diet with *Artemia* from hatch. However, there were technical difficulties associated with this. The nutritional value of rotifers and *Artemia* nauplii deteriorates quickly in clear water culture systems (Dhert 1996; Van Stappen 1996). Uneaten live food thus needs to be removed from the system, preferably on a daily basis. Rotifers are usually provided as adults and are easy to flush from the system through small sized outlet screens. However, even without food, *Artemia* nauplii moult to the second instar after about 8 hours and continue growing (Van Stappen 1996). Within 24 hours the metanauplii are equivalent in size to Z1 mud crab larva and are thus difficult to remove without losing the larvae through the appropriately sized outlet screens. Since there was no significant difference in performance for larvae fed *Artemia* at 1 or 4 DAH, the feeding of *Artemia* could be delayed until the larvae reached the Z2 stage and were

large enough to be retained by 300 µm screens. The advantage of delaying *Artemia* feeding to the Z3 stage is that the larvae were large enough to feed on enriched metanauplii. The benefit of providing both enriched rotifers and *Artemia* was demonstrated in chapter 7.

Small quantities of larvae fed decapsulated *Artemia* cysts from hatch survived through the zoeal stages, indicating that *S. serrata* accepted inert diets (Chapter 5). Formulated larval feeds are convenient and sterile. Small quantities of formulated feed are used as a supplement to live food for large scale rearing in the Philippines (Quinitio *et al.* 2001). Micronutrients can also be incorporated at desired levels and as opposed to live feeds (Evjemo *et al.* 1997; Han *et al.* 2001) can be delivered to the larvae unaltered. The use of formulated feeds has contributed significantly to our understanding of larval penaeid shrimp nutrition and a system similar to the one described by Jones *et al.* (1997) needs to be developed for mud crab larvae. Until such a system is developed, the nutritional requirements of the larvae must be investigated through the enrichment of live feeds through bioencapsulation.

Larval survival was not enhanced by enriching *Artemia* with several different (n-3) fatty acid emulsions or by providing larvae with a different strain of *Artemia* (sourced from Vin Chau, Vietnam) which contained high FAME and EPA levels (Chapter 7). Similar findings were recorded in Australia (Mann 2001; Graham Williams, pers. comm.). However, research in Japan (Kobayashi *et al.* 2000; Takeuchi *et al.* 2000) and recent studies in Vietnam (Truong Trong Nghia, pers. comm.) has shown that enriching *Artemia* with (n-3) HUFA does influence the larvae. It is possible that *Artemia* need to be enriched with nutrients other than (n-3) HUFA (Mann *et al.* 2001) and only continued research will determine whether enriching *Artemia* nauplii makes a significant enough difference to the larvae considering the time and investment involved in enriching *Artemia* on a commercial scale. Results demonstrated a significant benefit to enriching rotifers and as the nutritional profile of rotifers can be manipulated during production (Dhert 1996), focusing on the delivery of essential nutrients in this way may be more appropriate.

### **Analysis and recommendations**

High survival rates to megalopa could be repeatedly achieved in the small scale rearing system. However, high mortality and variable survival (particularly during the latter stages of development) were features of larger scale cultures. The project thus fell short of developing reliable technology for commercial seed production of mud crabs for aquaculture. The achievements of the project must however be seen in the light of similar projects elsewhere and

the conditions under which the project was undertaken in South Africa. As opposed to South Africa, countries in South East Asia have a long history of aquaculture and mud crab hatchery research in the region was initiated years before the South African project began. The projects in Australasia have been conducted with private and government backing in established marine research facilities with a team of qualified personnel. There has been in-depth cooperation between countries within the region for the last decade. However, only the group working in Darwin, Australia claims to have developed techniques suitable for commercial application and the causes of larval mortality are still poorly understood.

Despite the numerous obstacles, significant progress was made during the project. Toward the end, sufficient numbers of megalopae were being produced to initiate research on rearing systems for megalopae and small numbers of cultured juveniles (approximately 1500 per month) were released into a grow-out pond at MPF during the last six months of the project. Scientific results generated were on a par with those being produced in other parts of the world. The project proved that by applying standard larval rearing technology, significant progress in mud crab larval rearing can be made. Unlike panilurid lobsters for which a yet undiscovered breakthrough is probably required to establish commercial seed production, (Kanazawa and Koshio 1994; Kittika 1994; Jeffs and Hooker 2000), the answer to successful mud crab larval rearing lies in a sustained research effort which addresses all the relevant aspects.

Continued research and development will undoubtedly manage to establish hatchery technology for mud crabs in South Africa. However, suitable conditions must be in place if this is to occur within a reasonable time frame. A dedicated marine research laboratory is required, either close to a source of wild broodstock or with facilities to produce domestic broodstock from ponds. Key areas of research must include broodstock domestication and nutrition; zootechnics; microbiology and the investigation of probiotics and larval nutrition. A team of scientists, backed up by a technician dedicated to researching each of these aspects are required. Although mud crab hatchery technology is approaching commercial viability, it is not known when this will occur. It would be prudent to wait until the technology is established overseas so that local development can be augmented with technology transfer. Close cooperation between the private sector and research institutions was a major factor contributing to the success of abalone farming in South Africa (Sales and Britz 2001). Similar cooperation is needed for developing mud crab aquaculture.



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# Summary

South Africa has a small, but dynamic aquaculture industry which would benefit from diversification, especially through the introduction of high value species for export. The mud crab (*Scylla serrata*) is indigenous to the country, has a high market value, is suited to culture and has been identified as candidate species for aquaculture in the region. The bottleneck to the expansion of mud crab farming worldwide is the dependence on wild caught juveniles and adults for growing out to market size or fattening. Densities of mud crabs in South African estuaries are too low upon which to base a fishery or aquaculture industry. The establishment of hatchery techniques is therefore a prerequisite to developing farming in the country. The thesis describes research that was conducted to develop techniques required for egg production and larval rearing of *Scylla serrata* for aquaculture in South Africa.

Mud crab larvae were first reared over 40 years ago and research has intensified during the last decade. However, little of this work or other aspects of mud crab farming has been published in peer reviewed journals and much of it remains unknown to the scientific community. To address this shortcoming a review of all possible sources of literature was undertaken and unpublished information was collated to depict the current global status of mud crab hatchery technology. Aspects including techniques for broodstock acquisition, maturation, spawning, egg incubation and hatching are described. Larval rearing techniques such as stocking, water quality requirements, culture systems, feeding and nutrition as well as techniques for rearing the megalopae to juvenile crab are also described. From this review it was concluded that although some research groups have developed techniques for mass production of juveniles, much still needs to be done before seed production for aquaculture becomes economically viable. Major causes of mortality are disease and (probably) inferior nutrition. Research on probiotics, immunostimulants, formulated larval diets and live food enrichment is therefore required.

Prior to undertaking any experimental work it was necessary to establish whether South African *S. serrata* belong to one genetically homogeneous group or whether they vary between regions or between estuaries within regions. Mud crab populations were obtained from six estuaries in South Africa and one estuary in Madagascar. Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was used to assess the genetic variation

between the populations. A high level of variation with a low geographic bias suggested that the South African population should be considered a single entity but that the population from Madagascar is probably distinct. This implied that mud crab juveniles recruit into South African estuaries from a common regional pool. Although results must still be verified by a larger study using validated polymorphic microsatellite DNA markers, this has implications for aquaculture in the region. It is likely that the genetic homogeneity extends into Southern Mozambique and implies that potential collaboration between South Africa and Mozambique (where large areas of mangroves could be exploited for mud crab aquaculture) will probably not be limited by genetic differences between mud crab populations in the two countries. Care should however be taken to prevent genetic drift or impoverishment and not to compromise the genetic integrity of wild populations by translocation of juveniles for aquaculture, ranching or stock enhancement.

Establishing hatchery technology for new aquaculture species requires a regular and consistent supply of fertilised eggs. Information about broodstock availability and breeding biology is essential to achieve this goal. Mature female crabs were caught in the Umlalazi estuary, on the subtropical north-east coast of South Africa and held in a recirculating facility until spawning. Data was collected on catch per unit effort, crab size and mass. Spawning characteristics including time in captivity prior to spawning, batch and relative fecundity, individual egg mass and size, size of zoea 1 larvae, incubation time, hatch success rate, and seasonality and pattern of spawning were recorded. Of the 119 crabs kept in captivity, 83 % spawned in the maturation system - most within 40 days of capture. The crabs were highly fecund (mean relative fecundity per batch =  $10\,655 \pm 4,069$  eggs/g female). Spawning in captivity occurred throughout the year, with a peak in late winter/early spring. The high fecundity and the fact that the females spawned throughout the year, implies that relatively low numbers of wild-caught females are required for stocking hatchery operations. The crabs were easy to maintain, mature and spawn in captivity which will facilitate future domestication and will eventually reduce the need for wild caught broodstock. The spawning characteristics of South African *Scylla serrata* are similar to those observed for the genus throughout its distribution implying that husbandry, conservation and fisheries management could be similar to that established elsewhere.

Numerous factors affect the survival of cultured mud crab larvae. One of the most important aspects for any cultured species is feeding and nutrition. Experiments were designed and conducted to establish a suitable feeding regime for the larvae. Mud crab larvae can consume a variety of feeds including rotifers and *Artemia* nauplii. First feeding is an important

phase in the life history of marine larvae and the optimal first food for South African mud crab larvae was not known. In order to investigate this, larvae were fed either rotifers (*Brachionus* sp.); newly-hatched EG® (INVE Aquaculture, Belgium) type *Artemia* nauplii; newly-hatched Vinh Chau (Vietnam) *Artemia* nauplii (smaller strain); decapsulated cysts of EG® type *Artemia*; or decapsulated cysts supplemented with low densities of EG® type *Artemia* nauplii. Although results showed that it is possible to rear *S. serrata* larvae through metamorphosis on *Artemia* nauplii exclusively, larval performance (development, survival and successful metamorphosis) was significantly improved by the inclusion of rotifers as a first feed. No significant difference in performance was recorded between larvae fed on the two strains of *Artemia* nauplii. The performance of larvae fed on decapsulated cysts was relatively poor, but there were indications that the cysts and other inert diets may have potential as supplements to live food in larval rearing.

Although rotifers are a preferred first food, the larvae require a larger food item at some stage of development in order to sustain good growth and survival. A series of experiments were conducted to determine the optimum time for weaning the larvae onto *Artemia* nauplii. In the first experiment, larvae were fed rotifers for the first eight days after hatching. *Artemia* nauplii were introduced either just after hatch, at 4 days after hatch (DAH) – during the second zoeal stage, or at 8 DAH – during the third zoeal stage. A control group of larvae was fed rotifers exclusively for 18 days until the completion of metamorphosis to megalopa. In the second experiment, the same four feeding schedules as in the first experiment were used as well as another group of larvae that were fed only *Artemia* nauplii throughout the rearing period. Similar results were recorded in the two experiments. Larvae fed *Artemia* just after hatch and at 4 DAH performed significantly better than those fed *Artemia* at 8 DAH and larvae fed only rotifers or only *Artemia* throughout. This was particularly evident when examining the proportion of zoeae 5, which successfully completed metamorphosis to megalopa. Results confirmed that rotifers are needed as a first food, but that rotifers alone cannot be used to rear the larvae through metamorphosis. Poor performance of larvae fed on *Artemia* at 8 DAH, suggested that the diet should be supplemented with *Artemia* before the end of the zoea 3 stage. Although the larvae benefited from *Artemia* supplementation during the zoea 1 stage, when applying this feeding schedule to larger scale systems, it is suggested that *Artemia* be offered to *S. serrata* larvae after moulting to the zoea 2 stage.

The nutritional requirements of mud crab larvae are not known. Enriching the live food of crustacean larvae (including crabs) with highly unsaturated fatty acids (HUFA) has significantly improved growth and survival. A series of experiments were conducted, to

determine whether this was the case for South African *S. serrata*. In the first two experiments, the performance of larvae fed unenriched *Artemia* nauplii in a control was compared with larvae fed *Artemia* enriched with algae and two different commercial emulsions. In the third experiment, larvae in the three treatments were fed unenriched rotifers and then *Artemia* enriched with three experimental emulsions containing different levels of (n-3) HUFA. In the fourth experiment, larvae in three treatments were either fed enriched (with a commercial emulsion) rotifers, enriched *Artemia*, or enriched rotifers and enriched *Artemia*. Larvae in the control received unenriched live food. No significant differences in development or survival were recorded for larvae in any of the treatments in the first three experiments. In the fourth experiment, performance of the larvae was significantly improved when both rotifers and *Artemia* were enriched. The results suggest that enriching only the *Artemia* has little benefit for the larvae. In order for enrichment to be effective, both the rotifers and the *Artemia* must be enriched with HUFA.

Numerous other techniques were applied, adapted and developed for mudcrab larval rearing during the project. Towards the end of the project, sufficient numbers of megalopae were produced to initiate research on rearing systems for megalopae and several thousand cultured juveniles were released. The project has contributed towards the development of larval rearing protocols for *S. serrata*. Future work now needs to focus on broodstock domestication and nutrition to improve egg and larval quality; larval nutrition to improve growth and reduce moult death syndrome; and zootechnics, microbiology and the investigation of probiotics to reduce disease.

# Samenvatting

Zuid-Afrika heeft een kleine, maar dynamische aquacultuur industrie die gebaat zou zijn met diversificatie, vooral door introductie van soorten met een hoge exportwaarde. De mangrove krab (*Scylla serrata*) is inheems, heeft een hoge marktprijs, is geschikt voor kweek en werd naar voor geschoven als kandidaat soort voor de regio. Het belangrijkste probleem voor de expansie van de kweek van mangrove krab wereldwijd is de afhankelijkheid van in het wild gevangen juvenielen en adulten voor verdere opkweek tot marktgrootte. De densiteit van mangrove krabben in de Zuid-Afrikaanse estuaria is te laag om er een visserij of aquacultuur industrie op te baseren. Het verwezenlijken van broedhuistechnieken is daarom een noodzakelijke voorwaarde om kweek in het land te ontwikkelen. De thesis beschrijft onderzoek naar het ontwikkelen van technieken voor het verkrijgen van eitjes en larvale kweek van *Scylla serrata* voor de aquacultuur in Zuid-Afrika.

Larven van de mangrove krab werden het eerst gekweekt meer dan 40 jaar geleden en het onderzoek intensifieerde het laatste decennia. Weinig van dit werk of studies over andere aspecten van de kweek werden echter gepubliceerd in de vakliteratuur en blijven daarom onbekend bij de wetenschappelijke gemeenschap. Om hieraan tegemoet te komen, werd een grondige studie gedaan van alle mogelijke literatuurbronnen en samengebracht met niet-gepubliceerde informatie om een beeld te vormen van de huidige globale status van de broedhuistechnologie van de mangrove krab. Verschillende aspecten, waaronder technieken voor het verkrijgen van broeddieren, maturatie, ei-afleg, incubatie van de eitjes en ontluiking worden hierin beschreven. Technieken voor de kweek van larven, zoals het stockeren, waterkwaliteitsvereisten, kweeksystemen, voeding en nutritie, alsook technieken voor de kweek van het megalopa stadium tot juveniele krab worden eveneens besproken. Uit deze literatuurstudie kon geconcludeerd worden dat, hoewel sommige onderzoeksgroepen technieken hebben ontwikkeld voor de grootschalige kweek van juvenielen, er nog veel werk is vooraleer deze technieken ook economisch leefbaar worden. Belangrijke oorzaken voor sterfte zijn ziekte en (waarschijnlijk) suboptimale nutritie. Onderzoek naar probiotica, immunostimulanten, geformuleerde voeders en aanrijking van levend voedsel zijn daarom aangewezen.

Vooraleer het experimenteel werk aan te vatten, was het nodig uit te maken of de Zuid-Afrikaanse *S. serrata* tot één genetisch homogene groep behoorden. De alternatieve situatie zou

erin bestaan dat er genetische verschillen waren tussen de populaties van verschillende regio's, of tussen de populaties van verschillende estuaria binnen een regio. Monsters van mangrove krab populaties werden verkregen van 6 Zuid-Afrikaanse estuaria en 1 estuarium in Madagaskar. RAPD-PCR (Randomly amplified polymorphic DNA) werd gebruikt om de genetische variatie tussen de populaties te bepalen. Een hoog niveau van variatie gepaard gaand met een lage geografische variabiliteit suggereerden dat de Zuid-Afrikaanse populaties als één entiteit mogen beschouwd worden, maar dat de populatie van Madagaskar waarschijnlijk genetisch verschillend is. Dit geeft aan dat mangrove krab juvenielen in de Zuid-Afrikaanse estuaria rekruteren vanuit een gemeenschappelijke regionale pool. Hoewel deze resultaten nog moeten geverifieerd worden door een grotere studie gebruikmakend van gevalideerde hoogvariabele merkers (zoals microsatellieten), hebben ze nu al implicaties voor de aquacultuur in de regio. Het is waarschijnlijk dat de genetische homogeniteit doorloopt tot in zuiden van Mozambique, wat inhoudt dat de potentiële samenwerking tussen Zuid-Afrika en Mozambique (waar grote mangrove gebieden kunnen uitgebaat worden voor de aquacultuur van mangrove krab) waarschijnlijk niet zal gehinderd worden door genetische verschillen tussen de mangrove krab populaties in beide landen. Er moeten echter voorzorgsmaatregelen genomen worden om genetische drift of verarming te voorkomen om op die manier de genetisch integriteit van de wilde populaties niet te compromitteren door translocaties van juvenielen voor aquacultuur.

Het uitwerken van broedhuistechnieken voor nieuwe aquacultuur species vergt een regelmatige en betrouwbare voorziening van bevruchte eitjes. Informatie over beschikbaarheid aan broeddieren en voortplantingsbiologie is essentieel om dit doel te bereiken. Geslachtsrijpe vrouwelijke krabben werden gevangen in het Umlalazi estuarium aan de subtropische noordoost kust van Zuid-Afrika en gehouden in een recirculatie installatie tot ei-afleg. Gegevens werden verzameld over vangst per eenheid visserij-inspanning en grootte en gewicht van de krabben. Data over ei-afleg, zoals de tijd in gevangenschap vooraleer af te leggen, totale en relatieve fecunditeit, individueel gewicht en grootte van de eieren, afmeting van de zoea 1 larven, incubatietijd, ontlukingssucces, de invloed van het seizoen en patronen bij de ei-afleg werden verzameld. Van de 119 krabben die in gevangenschap werden gehouden, legde 83 % af in het maturatiesysteem – waarvan de meeste binnen de 40 dagen na vangst. De krabben waren zeer vruchtbaar (gemiddelde relatieve fecunditeit per afleg =  $10,655 \pm 4,069$  eieren/g vrouwtje). Ei-afleg in gevangenschap greep het hele jaar door plaats, met een piek in de late winter tot het vroege voorjaar. De hoge fecunditeit en het feit dat afleg het hele jaar door plaats greep, houden in dat maar relatief kleine aantallen in het wild gevangen vrouwtjes nodig zijn om broedhuizen van larven te voorzien. De krabben waren gemakkelijk te kweken en te matureren en legden

gemakkelijk eitjes af in gevangenschap. Dit impliceert dat domesticatie in de toekomst mogelijk zal zijn, hetgeen uiteindelijk de nood aan wilde broeddieren zal doen verminderen. De voortplantingseigenschappen van de Zuid-Afrikaanse *Scylla serrata* komen goed overeen met deze vastgesteld voor het genus over zijn volledige verspreiding en geven aan dat fokkerij, conservatie en visserijbeheer wellicht gelijkaardig kunnen zijn aan deze opgemaakt op andere plaatsen.

Heel veel verschillende factoren bepalen de overleving van mangrove krab larven in gevangenschap. Eén van de meest belangrijke aspecten voor iedere gekweekte soort is voeding en nutritie. Experimenten werden uitgevoerd om een geschikt voederschema voor de larven te bepalen. Mangrove krab larven kunnen een variëteit aan voedsel, waaronder rotiferen en *Artemia* nauplii, opnemen. De initiële voeding is een belangrijke fase in de levensontwikkeling van mariene larven en het optimale eerste voeder voor Zuid-Afrikaanse mangrove krab larven was niet gekend. Om dit te onderzoeken, werden larven ofwel gevoederd met rotiferen ( *Brachionus* sp.); pas-ontloken EG® (INVE Aquaculture, België) type *Artemia* nauplii; pas-ontloken Vinh Chau (Vietnam) type *Artemia* (type met kleinere afmeting); gedecapsuleerde cysten van het EG® type *Artemia*; of gedecapsuleerde cysten gesupplementeerd met lage densiteiten EG® type *Artemia* nauplii. Hoewel de resultaten aantoonde dat het mogelijk is *S. serrata* larven te kweken tot na de metamorfose met enkel *Artemia* nauplii, werd de prestatie (ontwikkeling, overleving en metamorfose succes) significant verbeterd door ook rotiferen in het eerste voeder op te nemen. Er werd geen significant verschil waargenomen tussen de larven gevoed met de twee types *Artemia*. De prestatie van de larven gevoed met gedecapsuleerde cysten was eerder laag, maar er zijn indicaties dat cysten of andere inerte voeders mogelijkheden zouden kunnen hebben als supplement voor levend voedsel in de kweek van de larven.

Hoewel rotiferen het meest geschikte eerste voedsel zijn, hebben de larven op een bepaald moment in hun ontwikkeling een grotere prooi nodig om goede groei en overleving te bestendigen. Een reeks experimenten werd uitgevoerd om te bepalen wat het beste tijdstip is om over te schakelen op het voederen van *Artemia* nauplii. In het eerste experiment, werden alle larven gedurende de eerste 8 dagen gevoederd met rotiferen. *Artemia* nauplii werden ofwel geïntroduceerd direct na ontluiking, of 4 dagen na ontluiking (DAH) – tijdens het tweede zoea stadium, of na 8 dagen – tijdens het derde zoea stadium. Een controle groep werd de volledige 18 dagen tot na de metamorfose tot megalopa enkel gevoed met rotiferen. In het tweede experiment, werden dezelfde 4 voederschema's toegepast, alsook een groep larven die enkel *Artemia* toegediend kregen tijdens de volledige kweekduur. Gelijkaardige resultaten werden

bekomen voor beide experimenten. Larven die gevoederd werden met *Artemia* direct na ontluiking en op DAH 4, deden het significant beter dan deze die pas gevoederd werden met *Artemia* op DAH 8 en de larven die enkel rotiferen of *Artemia* kregen gedurende de volledige periode. Dit was vooral duidelijk wanneer gekeken werd naar het aandeel zoea 5 larven dat succesvol metamorfoseerde tot megalopa. De resultaten bevestigden dat rotiferen noodzakelijk zijn als eerste voedsel, maar dat deze niet voldoende zijn als enig voedsel om een goede metamorfose te bekomen. De zwakke prestatie van de larven die pas vanaf DAH 8 met *Artemia* gevoederd werden, suggereren dat het dieet vóór het einde van het zoea 3 stadium met *Artemia* moet gesupplementeerd worden. Alhoewel de larven reeds voordeel halen bij supplementatie met *Artemia* in het zoea 1 stadium, wordt voor toepassing op grotere schaal toch voorgesteld pas vanaf de vervelling naar het tweede zoea stadium *Artemia* te voederen.

De nutritionele behoeften van mangrove krab larven zijn niet gekend. Aanrijking van het levend voedsel van larven van kreeftachtigen (waaronder krabben) met hoog onverzadigde vetzuren (HUFA) heeft gezorgd voor een significante verbetering van de groei en overleving. Een serie experimenten werd uitgevoerd om te onderzoeken of dit ook het geval is voor Zuid-Afrikaanse *S. serrata*. In de eerste twee testen werd de prestatie van larven die gevoederd werden met niet-aangerijkte *Artemia* (controle) vergeleken met larven die gevoederd werden met *Artemia* aangerijkt met algen en twee commerciële emulsies. In het derde experiment, werden larven van de drie behandelingen eerst gevoederd met niet-aangerijkte rotiferen en daarna met *Artemia* aangerijkt met drie experimentele emulsies met een verschillend (n-3) HUFA gehalte. In het vierde experiment werden de larven in de drie behandelingen ofwel gevoederd met rotiferen aangerijkt met een commerciële emulsie, ofwel met aangerijkte *Artemia*, of zowel met aangerijkte rotiferen als aangerijkte *Artemia*. Larven in de controle kregen niet-aangerijkt levend voedsel. Er werden geen significante verschillen in ontwikkeling of overleving vastgesteld tussen geen enkel van de behandelingen in de eerste drie experimenten. In het vierde experiment werd de prestatie van de larven significant verbeterd als zowel aangerijkte rotiferen als aangerijkte *Artemia* gebruikt werden. De resultaten tonen aan dat enkel aanrijking van de *Artemia* de larven weinig baat. Opdat de aanrijking effectief zou zijn, moeten zowel de rotiferen als de *Artemia* aangerijkt worden met HUFA.

Ook een hele reeks andere technieken voor de larvale kweek van mangrove krab werden ontwikkeld, , aangepast en toegepast tijdens het project. Naar het eind van het project werden voldoende aantallen megalopae geproduceerd om onderzoek te starten naar kweeksystemen voor megalopae en verschillende duizenden gekweekte juvenielen werden uitgezet. Het project heeft bijgedragen tot het ontwikkelen van protocollen voor de larvale kweek van *S. serrata*.

Belangrijke onderzoeksdomeinen voor de toekomst zijn de domesticatie van broeddieren en de invloed van nutritie op kwaliteit van eieren en larven; invloed van larvale nutritie op larvale groei en vervellings dood syndroom; zootechnische factoren en microbiologie en onderzoek naar probiotica om ziekte te verminderen.