AN INVESTIGATION INTO THE CAPTIVE SPAWNING, EGG CHARACTERISTICS AND EGG QUALITY OF THE MUD CRAB (*Scylla serrata*) IN SOUTH AFRICA

A thesis submitted in fulfillment of the requirements for the degree of

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Finally thanks to my parents who, without their support and understanding I would not be here today.

Abstract

The source of a reliable supply of seed is one of the most important factors determining the success of aquaculture. At present mud crab culture is reliant on wild caught juveniles, which limits the expansion of mud crab farming globally. This, coupled with the paucity of knowledge of the spawning and egg characteristics of mud crabs, prompted the investigation into the captive spawning, incubation and egg quality of *Scylla serrata* in South Africa.

A total of 112 mature females were caught from three estuaries in northern KwaZulu Natal and acclimated to captive conditions. Mature females were present in the estuaries throughout the year and ranged from 90 to 200 mm carapace width. Except for 40 crabs all the females spawned in captivity. On average spawning took place 38 ± 23 days after capture but was not dependent on crab size. Fecundity was significantly correlated to crab size ($p = 0.026$), with larger females producing more eggs. The average fecundity per batch was 5.79 ± 2.07 million eggs. Hatch success rate for all egg batches was high averaging 84 ± 6 %. Egg dropping occurred in all egg batches during incubation. On average 6.5 % of the egg mass was dropped during incubation. At the average temperature of 27^{o}C eggs in the incubation system hatched within 292 ± 12 hours after extrusion. In-vitro incubation of eggs proved to be an ineffective means of incubating *Scylla serrata* eggs with a hatch success rate of only 25 ± 5 %.

Egg quality was assessed both directly, using variables such as hatch success rate and hatch synchronicity and retrospectively by using stress tests and comparing survival of

larvae from different coloured egg batches. Lipid class composition and fatty acid methyl ester content for 28 batches of eggs and 16 batches of larvae were analyzed. The biochemical composition of the egg was correlated to survival of larvae in the stress tests to ascertain if the lipid content of the eggs determines their quality. Stress tests were used to retrospectively assess egg quality. Four stressors were administered to newly hatched larvae and the LD_{50} values recorded. An average LD_{50} of 64 hours was recorded for the starvation tests, 40‰ for the salinity tests, 37 ppm for the formalin tests and 39.7 mg/l NH_3 for the ammonia stress tests was recorded.

Egg colour ranged from pale yellow to orange-red. Egg colour was not influenced by female size or the time the females spent in captivity prior to spawning. Egg colour can therefore not be used as an indicator of quality. The lipid profiles of newly extruded eggs had no effect on hatch synchronicity or hatch success rate. However, the stress tests identified female size ($p \leq 0.02$), DHA content of the egg ($p \leq 0.02$), Σ omega-3 fatty acids of eggs ($p \leq 0.02$) and EPA content of eggs ($p \leq 0.007$) as possible determinants of egg quality. Larger crabs tended to produce poorer quality eggs. Egg quality also decreased as the amount of DHA, EPA and the Σ omega-3 fatty acids decreased in the eggs. To summarize, the results of this study indicated that the following parameters affect egg quality - crab size, DHA, EPA and Σ omega-3 fatty acid content of eggs.

Chapter 1

General Introduction

Prior to Keenan *et al.*'s (1998) revision of the genus *Scylla*, all the work on the biology and culture of mud crabs was based on Stephenson and Campbell's (1960) monospecific classification of the genus. Stephenson and Campbell (1960) suggested that there was insufficient evidence for the separation of species beyond *S. serrata* (Forskal) (Le Vay 2001). Keenan *et al.* (1998), however, separated the genus using genetics and morphology into four distinct species, consisting of *S. serrata, S. paramamosain, S. olivacea* and *S. tranquebarica*. Of the four, *S. serrata* is the largest and has the widest distribution, occurring throughout the Indo-West Pacific, Red Sea, Australia, Philippines, Taiwan, Japan, South Africa and the Pacific Islands (Keenan *et al.* 1998). Keenan's revision of the genus *Scylla* has rendered much of the historical information on biology, fisheries and aquaculture redundant, especially in areas where more than one species occurs. This makes it difficult to draw inferences from past literature, as much of the biological and ecological data available will have to be reassessed (Le Vay 2001).

Scylla species commonly inhabit sheltered estuaries, mud flats and mangrove forests (Hill 1974, Robertson 1989, Le Vay 2001). The distribution of crabs within these habitats appears to be dependent on the stage of ontogenetic development. Juveniles, up to 8 cm carapace width, are more abundant in the intertidal mud flat habitats within estuaries while adult crabs are more abundant in the sub tidal regions of estuaries (Le Vay 2001). *S. serrata* feed on a variety of food items. Juveniles, which are more mobile, feed on prawns, smaller crabs, fish and other small invertebrates (Joel and Sanjeevaraj 1986). Large adult crabs have unspecialised diets and are best described as opportunistic omnivores (Warner 1977). The diets of larger crabs include molluscs, carrion, prawns, submerged aquatic weeds and detritus (Hill 1979, Joel and Sanjeevaraj 1986, Paterson and Whitfield 1997).

Mating takes place in the estuarine environment, after which female crabs migrate to the sea where spawning takes place (Arriola 1940, Ong 1966). Berried *S. serrata* females have been caught in trawl nets up to 80 km from the shore in Australia (Poovichiranon 1992, Hill 1994). Spawning appears to occur throughout the year with some seasonal peaks (Heasman 1980, Quinn and Kojis 1987). These peaks seem to be related to seasonal rainfall for tropical populations, while in temperate regions reproduction is more strongly related to temperature, with a peak in spawning activity in the summer months (Heasman *et al.* 1985). *S. serrata* is highly fecund with up to 8.36 million eggs per female (Mann *et al.* 1999). The zoeal larvae develop and remain in the open ocean until they reach the megalopa stage, after which they migrate back into the estuarine environment (Keenan 1999). Little is known about the oceanic phases of the life cycle (Fielder 1995).

Moulting and growth in *S. serrata* continues throughout the life cycle (Warner 1977). Size at sexual maturity for both females and males is known to vary widely, ranging from 83 – 144 mm carapace width (Hill 1975, Heasman *et al.* 1985). Growth rates are relatively fast and sexual maturity is attained within 12 months (carapace width 10.5 cm) in Papua New Guinea (Quinn and Kojis 1987) and between 12 to 18 months (carapace width 8.3-14.4 cm) in South Africa (Hill 1975). The fast growth rate coupled with high fecundity makes *S. serrata* an ideal candidate for aquaculture.

Scylla species are important in many small-scale coastal fisheries in sub-tropical and tropical Asia and Australia. Until the late 1980s the total volume of all crab species sold worldwide as a percent of crustacean sales was a mere 3.5 % (i.e. less than 10 000 tons) (FAO 1995). By 1993, the total volume of caught and cultured crabs reached almost 40 000 tons, 29.8 % of which were mud crab (FAO 1995). Due to their large size, high meat yield and delicate flavour, mud crabs are sought after as a quality food item (Keenan 1999). In Australia and Asia at present, the volume of mud crabs imported and exported remains relatively low due to the high local demand (Knuckey *et al.* 1995).

At present, mud crab aquaculture worldwide is dependent on wild juveniles. The limited supply of wild seed for stocking purposes (Keenan 1999) is a major constraint for the

development and expansion of mud crab farming (Liong 1992). A further constraint is the seasonal fluctuations in abundance of juveniles, making the supply of seed for aquaculture purposes unreliable (Robertson and Kruger 1994, Knuckey *et al.* 1995, Keenan 1999, Le Vay 2001). Although cultured crabs will compete with wild caught crabs in the market, it is generally believed that demand for both cultured and wild crabs exceeds supply worldwide. Aquaculture facilities can control production to maximise economic benefits by supplying product during periods when wild catches are low. In countries like Australia, where minimum legal size limits are set for wild caught crabs, farmed crabs may fill an important gap in the market for smaller, soft-shelled crabs which command up to three times the market price of larger legal sized crabs (Kennedy 1985).

At present, mud crabs are farmed in two ways, with both methods relying on captured animals. The first method consists of fattening thin, post-moult, "watery crabs" of marketable size. These are fattened for a 10 – 20 day period before being sold (Csavas 1995). These crabs generally range in size between 250 – 1000 g. The second culture method involves the grow-out of juveniles or undersized crabs, usually smaller than 250 g. The grow-out process typically lasts for 2 – 3 months, during which a significant increase in size occurs, with crabs moulting up to seven times (Csavas 1995, Keenan 1999).

Due to the high demand for mud crab in many Asian, Australian, American and European markets and the limited supply of wild seed, extensive research has been conducted on seed production (Keenan 1999). Research needs were first addressed and identified at the Regional Seminar on Mud Crab Culture and Trade in the Bay of Bengal region, held in Thailand in November 1991 (Angell 1992). The research needs include:

- Intensive research to be carried out on larval rearing techniques, including water quality, nutritional requirements of larvae, as well as broodstock maturation and spawning.
- Studies on nutrition, cannibalism, water quality, pond management and disease to improve survival during grow-out.

- A review of the systematics of *Scylla* species.
- To provide technical support for the mud crab trade, including packaging technology, market intelligence as well as extension and training programme to popularise mud crab culture.

Since this conference, three of the four issues have been thoroughly investigated. Survival rates now exceed 75% in grow-out facilities (Varikul *et al.* 1972, Cholik and Hanafi 1992, Macintosh *et al.* 1993). The systematics of the genus *Scylla* has been mapped out and individual species can now be accurately identified (Keenan *et al*. 1998). Numerous economic feasibility studies have been conducted that have identified markets for cultured mud crab (Agbayani *et al.* 1990, Brienl and Miles 1994, Aldon and Dagoon 1997). To date, however, larval rearing is still the principal bottleneck for the successful development of mud crab farming (Le Vay 2001).

Variable egg quality has been identified as one of the main limiting factors for the mass production of juvenile marine fish fry and crustaceans (Kjørsvik *et al.* 1990). The term "egg quality" has been defined and used differently by numerous authors, the most appropriate of which was adopted by Kjørsvik *et al.* (1990), and refers to "the egg's potential to produce viable fry". During the last 20 years, numerous studies on a variety of fish species (Kjørsvik *et al.* 1990, Bromage and Roberts 1995, Peleterio *et al.* 1995, Furutia *et al.* 2000, Morehead *et al.* 2001) and some crustaceans (Harrison 1990, De Caluwé *et al.* 1995_b, D'Abramo 1997, Smith *et al.* 2002) have investigated factors that influence egg quality (De Caluwé *et al.* 1995_b, Peletreiro *et al.* 1995, Furutia *et al.* 2000). Factors affecting egg quality are determined by the intrinsic properties of the egg itself, and the environment in which the egg is fertilized and subsequently incubated (Brooks *et al.* 1997). Factors that have been shown to affect egg quality include diet of the broodstock, endocrine status of the female during growth of the oocyte in the ovary, the complement of nutrients incorporated into the oocyte and the subsequent physiochemical conditions of the water in which the eggs are incubated (Brooks *et al.* 1997). Husbandry practices are probably the major contributing factor affecting egg quality in captive broodstock (Brooks *et al.* 1997).

Broodstock nutrition has been shown to affect egg quality in both fish (Lavens *et al.* 1999) and crustaceans (Lavens *et al.* 1991, Palacios *et al.* 1998). The accumulation of essential nutrients such as essential fatty acids and Vitamin C within the egg is dependent on the nutrient reserves of the mother and the diet in the period preceding gonadogenesis (Bell *et al.* 1997). Although broodstock nutrition has been identified as a factor affecting egg quality, its effects are masked by high data variability (Lavens *et al.* 1999).

To date, most studies dealing with the effects of diet on egg quality have focused on macro-nutrients i.e. proteins, fats and carbohydrates (Brooks *et al*. 1997). The effect of lipids and lipid composition on egg quality is the most extensively studied field in both fish (Harel *et al.* 1994, Carrillo *et al.* 1995) and crustaceans (Harrison 1990, Xu *et al.* 1994, Jones *et al.* 1997). It has been shown that the Σ omega – 3 fatty acids were particularly important, with eggs having higher levels of these fatty acids being of better quality (Jones *et al.* 1997). Few studies have been done on the "minor" dietary constituents, although some trace elements and vitamins have been linked to egg quality in fish (Hardy 1983). In crustaceans, the vitamin requirements have yet to be completely defined and this is extremely challenging (Conklin 1997).

It is well known that larval size is related to egg size and that larger larvae survive longer without food than those hatched from smaller eggs (Blaxter 1988, Kjørsvik *et al.* 1990, Richter *et al.* 1995). Bigger eggs have historically been regarded to be of better quality than smaller eggs (Brooks *et al.* 1997). It has been shown, however, that egg size has no direct implication on egg quality, in terms of larval survival and growth in a variety of fish species (Kjørsvik *et al.* 1990). One possible explanation for these findings is that egg size is highly variable in most species (DeMartini 1991), with egg size varying by up to 70 % in species such as the coho salmon (*Oncorhynchus kisutch*) (Brooks *et al.* 1997). Similarly in crustaceans, larger egg size is not an indicator of egg quality (Hines 1986). Instead it affects incubation time, the size of first larvae, duration of the planktonic period and the time to metamorphosis (Mileikovsky 1971, Hines 1986).

Good husbandry practices are critical for the production of good quality larvae (Bromage and Roberts 1995, Brooks *et al.* 1997). Such practices include optimising feeding regimens and food quality, maintaining optimum environmental conditions to reduce captive stress levels, and the control of pathogens and micro-organisms in the water. To date, the majority of research conducted on mud crabs has focused on larval rearing with little attention being paid to the maintenance of broodstock, spawning behaviour and the determination of egg quality.

Little is known of the captive spawning of *S. serrata* in South Africa. The aims of this study were to investigate and develop appropriate protocols for the capture and maintenance of wild broodstock, to develop an appropriate captive spawning environment (Chapter 2), to investigate spawning and egg characteristics of *S. serrata* (Chapter 3) and to determine which broodstock and egg parameters could be used as reliable indicators of egg quality (Chapter 4).

Chapter 2

General materials and methods, broodstock capture and holding

Introduction

In aquaculture, the source of a reliable supply of seed is one of the most important factors influencing the culture of a species. Internationally the culture of mud crabs is presently reliant on wild caught juveniles (Macintosh *et al.* 1993). Mud crab fisheries have declined in Taiwan (Chang and Wu 1985), Philippines (Aldon and Dagoon 1997), Thailand (Macintosh *et al.* 1993) and many other countries within the Indo-Pacific region (Escritor 1972, Varikul *et al.* 1972) and this has stimulated interest in the development of aquaculture practices for this species (Escritor 1972, Varikul *et al*. 1972, Heasman and Fielder 1983). This move towards aquaculture has highlighted the need to develop suitable methods of broodstock capture and systems in which to hold them until spawning. This chapter describes the methods used in this study to capture, transport, disinfect and feed the broodstock females, and the maturation system that was used to hold the females until they spawned.

Broodstock capture

Broodstock were collected from three estuaries in northern KwaZulu-Natal (KZN) viz. the Mlalazi ($31^{o}47$'E; $28^{o}57$'S), Matigulu ($31^{o}38$'E; $29^{o}05$'S) and Richards Bay ($32^{o}05$'E; $28^{o}47$'S). Crabs were caught using lift nets due to their ease of operation and the fact that one person could, using an inflatable boat, easily transport, set and check the traps (Figure 2.1). Traps were deployed in water ranging in depth from 0.5 - 1.5 m and checked every half-hour. The bait used in the traps consisted of sardine (*Sardinops sagax*), squid (*Loligo sp.*) and, when available, prawn heads (*Penaeus indicus*). All crabs caught in the traps were removed and sexed (Figure 2.2) and all females were kept in holding bins, for no longer than 8 hours until sampling was completed.

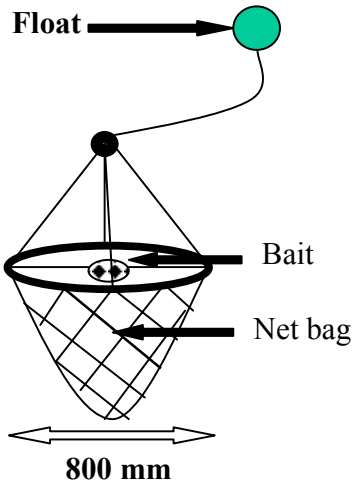


Figure 2.1: Schematic drawing of the lift-nets used to capture broodstock.

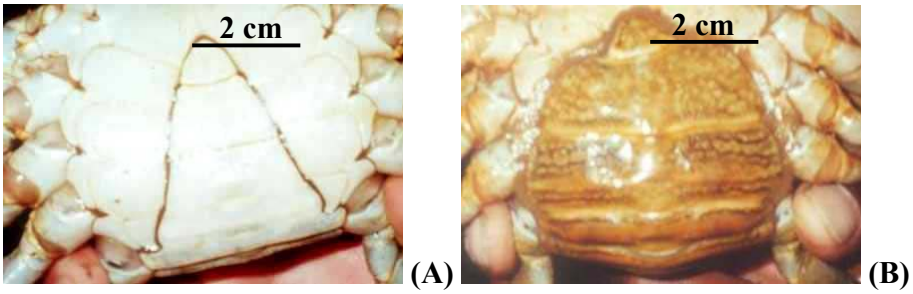


Figure 2.2. Abdomen of a male (A) and a female (B) mud crab, *Scylla serrata*

All male crabs were released back into the estuary and the remaining females were examined for sexual maturity (Figure 2.3). Females were identified as being mature by their wide, dark, U-shaped abdomen (Arriola 1940, Warner 1977, Heasman 1980, Barnes 1987) fringed with setae (Robertson and Kruger 1994). Immature females were characterised by having an abdomen resembling that of the male with slightly convex sides and no setae (Robertson and Kruger 1994). All immature females were returned to the estuary.

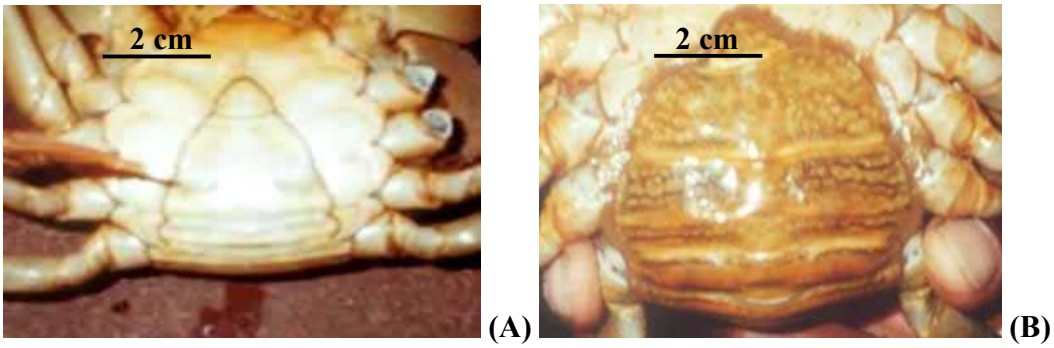


Figure 2.3. Abdomen of an immature female (A) and mature female (B).

Of the 112 mature female crabs stocked into the broodstock system, 84 were caught using lift nets. The other 28 females were obtained from recreational fishermen and from the prawn ponds on the farm during harvesting. Monthly catch per unit effort (CPUE) of mature females for the lift nets was calculated using the equation:

$$CPUE = \left(NC / ST\right) / NT$$

where NC = number of crabs caught

ST = soak time in hours

NT = number of traps

CPUE varied between months, ranging from 0.05 crabs per trap hour in January 2000 to 0.2 crabs per trap hour in March. During the rest of the year CPUE remained relatively constant at around 0.08 crabs per trap hour. Crabs were not sampled in October 2000 due to extreme weather conditions. The average CPUE obtained during the entire study was 0.08 ± 0.04 crabs per trap hour. This meant that one mature female crab was caught in each trap after a period of approximately 12.5 hours.

The sexually mature females ranged in size from 90 to 200 mm carapace width (CW). In total 94% of the females were larger than 120 mm CW (Figure 2.4). This is in agreement with the findings of Robertson and Kruger (1994), who estimated the size at 50 % sexual maturity for *Scylla serrata* in KwaZulu-Natal to be 123 mm.

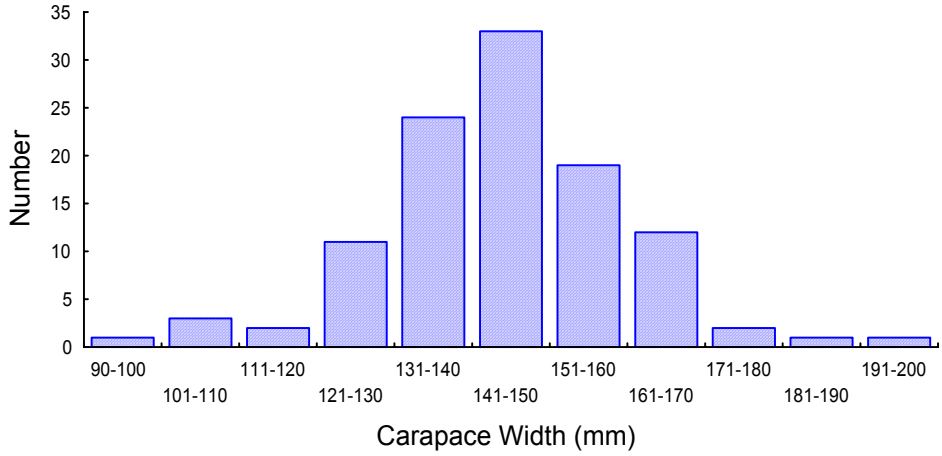


Figure 2.4: Size frequency distribution of mature female *S. serrata* captured in the Mlalazi, Matigulu and Richards Bay estuaries in KwaZulu Natal (n = 84)

The average size of mature females caught each month was relatively constant throughout the year with an average carapace width of 143 mm (Figure 2.5).

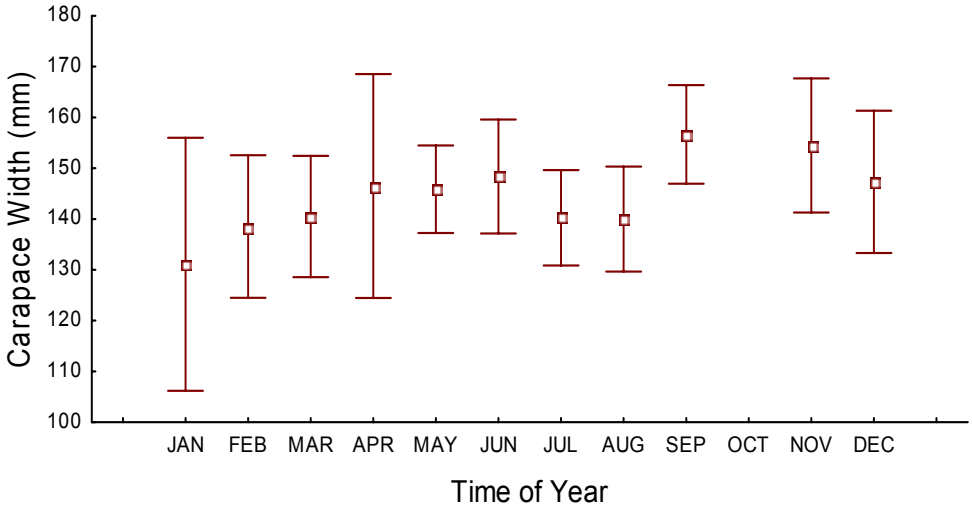


Figure 2.5: Carapace width of mature females caught from January 2000 to August 2001 (n = 84)

Several factors influenced the catch rate of crabs in traps. These were size and sex of the crab, temperature, trap spacing, soak time and time of day (Williams and Hill 1982, Robertson 1989). During this study catches were highly dependent on prevailing weather conditions and tidal changes within the estuaries. Catches were also low during extended

periods of rain and cold weather. Strong winds and tidal currents also affected catch rate by hindering the approach of the boat to the traps. Through observation a slow approach to the traps allowed the crabs sufficient time to let go of the bait and leave the traps as they were being pulled up.

Transport

The mature females were transported back to the lab in a 200 L plastic bin, filled with damp hesian sacking. Depending on sampling success, densities of crabs in the bin during transport were as high as 5 per m^2. Transport times never exceeded 35 minutes from site of capture to the laboratory. In other studies, broodstock crabs are often transported to the hatchery in 5 - 20 L of water for short journeys (> 30 minutes) and in large tanks supplied with aeration for long journeys (Rangneker and Deshmukh 1968, Heasman and Fielder 1983). In this study the use of water as a transport medium proved ineffective due to the condition of the roads.

No limb loss or mortalities were recorded in this study, suggesting that the use of the bin and damp sacking mode of transport was highly effective. The sacking kept the crabs cool as well as allowing them to crawl into the folds, minimising the contact between them. Once in a fold of sacking the crabs generally remained motionless for the duration of the trip back to the laboratory.

Disinfecting

Under culture conditions, the ability to control disease is vital because the potential for pathogen proliferation increases with the density of the cultured animals (Castille and Addison 1986). Formalin has been used extensively in crustacean culture for disinfecting and disease prevention (Chen *et al.* 1992, Primavera *et al.* 1993, GESAMP 1997, Chang *et al.* 1998, Brock and Bullis 2001). It was for this reason that all newly caught broodstock females were disinfected with formalin to reduce the number of symbionts and parasites found on the crabs before introducing them into the broodstock system.

Formalin has been used successfully in other studies to disinfect *S. serrata* broodstock (David Mann, Bribie Island Aquaculture Research Centre, Queensland, Australia. pers. comm., Lavina 1980). Formalin doses and exposure times vary widely between studies, with doses ranging from 100 ppm for 1 hour (David Mann, Bribie Island Aquaculture Research Centre. Queensland, Australia. pers. comm) to 50 ppm for 20 min (Lavina 1980). In this study all newly caught crabs were disinfected in an aerated 100 ppm formalin bath overnight for 12 hrs. Although this treatment was much higher and administered for longer than the doses used in the other studies mentioned above, the crabs showed no adverse affects to the dose.

Broodstock marking

A record of each crab was kept including the date introduced into the system, body mass and carapace width. Body mass was measured to the nearest gram and carapace width was measured, using vernier callipers, between the tips of the ninth antero-lateral spines (Robertson and Kruger 1994). For identification purposes, each crab was allocated a number in ascending order, which was engraved onto the carapace (Figure 2.6) using a battery powered hand held engraver. On average, the measuring and engraving of the crabs took approximately 5 min per crab.

Figure 2.6: Engraving of the carapace for identification.

The crabs showed no adverse effects to engraving of the carapace. In other studies, broodstock are marked in a variety of ways for identification purposes (Hill 1979, Ong 1966, Mann *et al.* 1999). The most common method is the attachment of a tag to the coax

of the swimming leg. This method of identification was tried initially but the crabs often physically pulled the tags off. The tags also seemed to interfere with their swimming ability. Those crabs that moulted in the maturation tank could be identified by reading the number off the shed carapace.

Broodstock maturation system

At present the life cycle for *S. serrata* has not been closed and currently all mature female crabs are caught from the wild and placed into maturation systems until they spawn (Ong 1966, Rangneker and Deshmukh 1968, Heasman and Fielder 1983, Robertson and Kruger 1994, Mann *et al.* 1999). Maturation systems vary widely between studies, ranging from flow-through systems (DuPlessis 1971), to recirculating systems (Hill 1980, Heasman and Fielder 1983, Mann *et al.* 1999) and stagnant systems with a complete daily water exchange (Djunaidah *et al.* 2001).

The maturation system used in this study was an indoor recirculating system, consisting of a 20 000 L bare bottomed cement maturation tank (20 m^2 and 1 m deep) and a 600 L sump that was linked to a 50 L mechanical sand filter and a 600 L biological filter. Air supply was provided by five air stones as well as the aeration caused by the inflow of water into the tank. All water entering the system was pumped from a header tank on site, which was continually kept full with fresh seawater. The operation of the system is presented schematically in Figure 2.7. The flow rate into the maturation tank resulted in a complete water exchange every 3.7 hrs or 6 times every 24 hours.

Water quality parameters were monitored at 3-day intervals throughout the study period. Dissolved oxygen, temperature and pH were tested using a Multiline P3 pH/Oxi-set while ammonium levels were tested using a Merck ammonium test kit. Dissolved oxygen concentrations were maintained at over 85 % saturation throughout the study period and pH ranged between 8.0 and 8.1. Un-ionized ammonia (NH_3) levels did not exceed 0.2 mg/l. The temperature in the maturation tank varied between summer and winter. Winter temperatures ranged from 22 – 25^oC, while summer temperatures ranged between 25 – 29

^{o}C (Table 2.1). Under natural conditions, temperatures in the Mlalazi estuary range between 17 – 22^{o}C in winter to 23 – 30^{o}C in summer (Robertson and Kruger 1994).

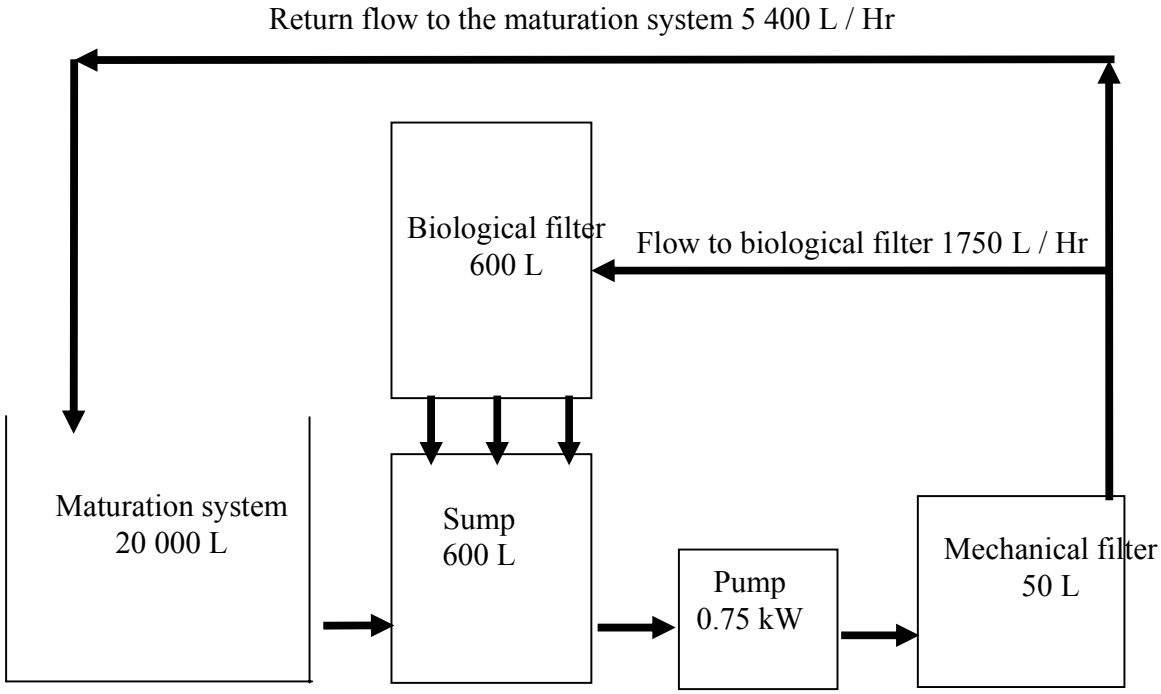


Figure 2.7: Schematic diagram of the water flow from the maturation system into the sump and then through the filters and back to the maturation system.

Table 2.1: Water quality parameters recorded in the broodstock system during the course of the study.

Water Quality Parameters	**Min**	**Max**	**Mean±S.D.**
Dissolved oxygen (%)	85	90	87±2.4
pH	8.01	8.12	8.06±0.04
Summer temperatures (^{o}C) November-February	25	29	27±1.5
Winter temperatures (^{o}C) June-August	22	25	23.±1
Un-ionized ammonia (NH_3) (mg/l)	0	0.2	***

The system was maintained under low artificial light conditions (1 - 3 Lux, Lutron LX-105 light meter) with the use of fluorescent tubes at a photoperiod of 9 hrs light and 15 hrs dark. The bottom of the maturation tank was cleaned daily by siphoning out uneaten food and waste. All screens were removed and cleaned daily. The use of a bare bottomed tank supplied with spawning bins allowed for the easy removal of uneaten food and faeces. On average, it took one person approximately half an hour a day to clean the maturation tank and filters.

Spawning substrate

The types of spawning substrates used for mud crabs vary widely between studies. At the Institute for Marine Aquaculture of Can Tho University in Vietnam, females are kept in individual 50 L sand bottomed bins connected to a recirculating system (Truong Nghia *pers. comm.*) Heasman and Fielder (1983) and Mann *et al.* (1999) kept crabs in bare bottomed tanks supplied with spawning trays and Djunaidah *et al.* (2001) kept crabs in tanks with mud or sand.

In this study, the crabs were provided with spawning bins consisting of plastic tubs with a diameter of 75 cm and a depth of 25 cm, and filled with beach sand to approximately 4 cm below the rim of the bin (Figure 2.8). Each bin was fitted with an airlift system.

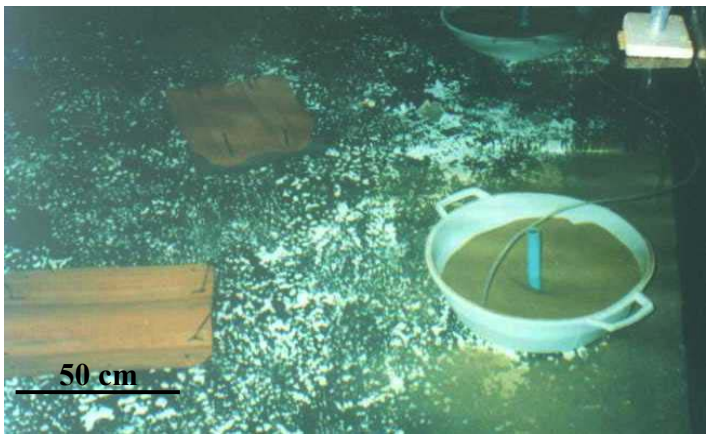
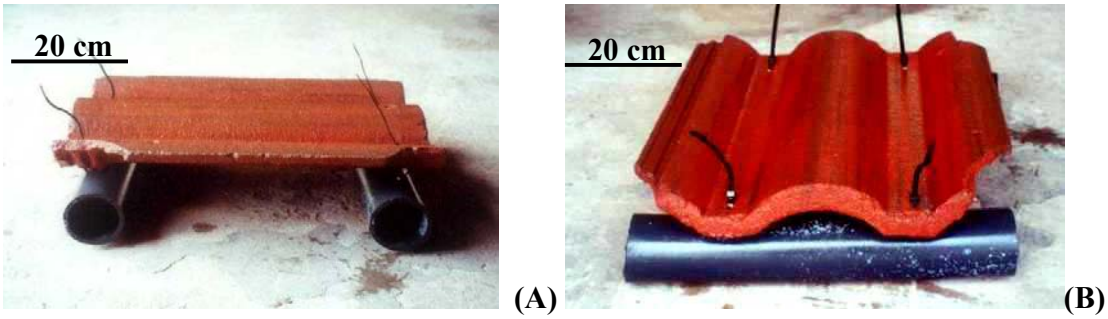


Figure 2.8: Sand filled spawning with airlift system in the maturation tank.

In this study, crab density within the maturation tank did not exceed 1.5 m^2 (Heasman and Fielder 1983, Marichamy and Rajapackiam 1991, Mann *et al.* 1999). Densities were kept purposefully low in order to reduce interactions between crabs due to their aggressive behaviour and tendency to cannibalise each other (Ong 1966, Lavina 1980). To further reduce aggressive encounters, shelters were provided and placed randomly within the maturation tank. The crabs took refuge under these shelters and thereby reduced contact with each other. The shelters consisted of roofing tiles, which rested on two short pieces of 50mm PVC piping (Figure 2.9).



(A) **(B)**

Figure 2.9: Front (A) and side (B) of the shelters provided in the maturation tank

Feeds and Feeding

Broodstock were fed on a mixed diet consisting of squid (*Loligo sp.*), prawns (*Penaeus indicus*), mangrove snails (*Cerithidea decollata*) and fish roe. Initially a combination of feed types was fed daily at a rate of approximately 10 % of the crab biomass. However, it was observed that if all feeds were offered simultaneously, prawn and squid were selected first, fish roe second and mussel meat was often ignored. The feeding regime was therefore changed to a rotational basis where one type of feed was fed daily. The crabs were fed at 09:00 in the morning and food levels checked again at 15:00 in the afternoon. If all the food had been consumed then more was added.

Conclusions

The use of baited lift nets was an effective and simple way to collect mature female crabs in KwaZulu-Natal estuaries. Catches were affected by adverse weather conditions such as cold weather, strong winds, tidal currents and temperature. Transporting the crabs from sampling site to the laboratory was best achieved in a bin containing damp hesian sacking, which reduced contact between crabs. Disinfection of the broodstock in a 100 ppm formalin bath for 12 hours overnight was effective and had no adverse effect on the crabs. Marking the crabs by engraving the carapace proved to be highly effective. The use of a bare bottomed cement maturation tank supplied with sand filled spawning bins enables easy maintenance and cleaning of the system, ensuring high water quality. Stocking crabs at a density of 1.5 m^2 and the provision of shelters ensured minimal contact and aggressive behaviour between crabs.

Chapter 3

Spawning, egg and hatching characteristics of mud crab

Introduction

Scylla serrata females migrate out of estuaries into the marine environment to spawn, where they remain until the eggs hatch (Hill 1975, Heasman and Fielder 1983, Hill 1994). This has made the collection of data on spawning behaviour and egg production very difficult. At present information on the reproductive biology of *S. serrata* in South Africa is restricted to ovarian maturation indices (gonado-somatic indices) from crabs caught in the wild (Roberts and Kruger 1994). Captive spawning of *S. serrata* in South Africa has been reported but observations were based on a limited (less than 10) number of crabs and were not the focus of the study (DuPlessis 1971, Robertson and Kruger 1994).

Scylla serrata, like most crustaceans, are dioecious and copulate in order to reproduce. The gonads of both male and female crabs are paired organs situated just under the carapace. In males the gonads are joined just posterior to the stomach while in the female the gonads extend back below the pericardium to join posteriorly. Short oviducts on each side of the female connect the spermathecae to the posterior lobes of the ovaries. Eggs are fertilized during their passage through these oviducts (Barnes 1987). The sizes of the gonads depend on the age of the crab and on the time of year (Pillai and Nair 1968, Warner 1977, Heasman 1980, Robertson and Kruger 1994). In immature females, the ovaries are small and only partly fill the body cavity. In mature females, however, the ovaries during the breeding season fill the entire body cavity (Figure 3.1)

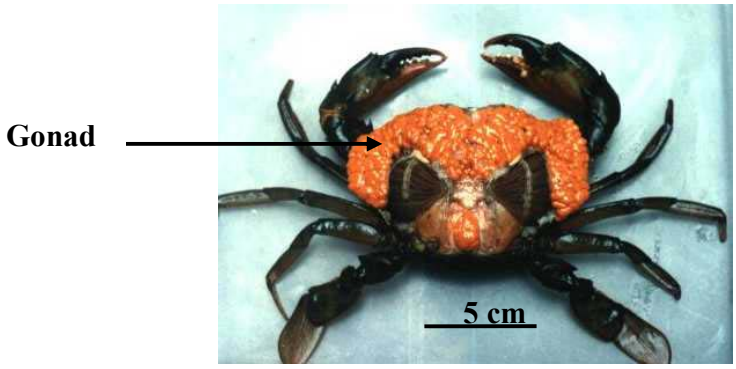


Figure 3.1: Dorsal view of ovaries of a mature female mud crab, *Scylla serrata* with the carapace removed.

The testes are smaller than the ovaries but are still easily discernable in mature animals. Most noticeable are the vasa deferentia, which emerge from the testes on either side of the stomach as a pair of thin white-coiled tubes. The vasa deferentia lead into ejaculatory ducts that open on the ventral side of the last pair of walking legs (8^{th} thoracic segment). In females, the paired genital openings are found on the sternite of the 6^{th} thoracic segment with each opening leading up a short duct into a sack-like spermathecae lying amongst the basal muscles of the second walking leg (Warner 1977, Bliss 1983, Barnes 1987).

Mating in crabs occurs while the female is in a soft-shelled state just after moulting (Hartnoll 1968, Barnes 1987). The male crab finds a receptive female that is ready to moult and clasps her with his walking legs and carries her beneath him. This position is termed the pre-copulatory embrace and continues until the female is ready to moult (Barnes 1987). In *Scylla serrata,* the pre-copulatory embrace lasts for approximately 3 - 4 days before moulting (Chen 1976). When the female begins to moult, the male releases her and guards her. Immediately after the female has completed moulting the male again clasps the female but with the female's abdomen up against the male's abdomen to facilitate mating. Copulation may last as long as 5 to 12 hrs (Chen 1976, Barnes 1987). Sperm is stored by the female in the spermathecae and can be retained even if the female moults (Bliss 1983).

Insemination must be distinguished from fertilization, as the two may be widely separated in time. The time between insemination and fertilization varies greatly between species (Berry 1970, Warner 1977). Spawning involves the fertilization of the eggs and the attachment of the eggs to the pleopods. Once the female has extruded the eggs they are cemented to the pleopods with a material associated with the egg membrane (Barnes 1987).

Several authors have investigated the spawning and reproductive performance of *Scylla* species in captivity to develop best management practices for captive breeding (Ong 1966, Brick 1974, DuPlessis 1971, Heasman and Fielder 1983). Much of the work that was published before the revision of the genus (Keenan *et al.* 1998) leaves room for speculation, as the actual species from which data were collected is not known. It is for this reason that this investigation was carried out on the South African population of *Scylla serrata*. Several parameters have been identified that may affect the reproductive performance of crustaceans. These include fecundity, egg size, incubation period, egg dropping and hatch success (Bromage and Roberts 1995). In order to quantify the effects that these parameters have on the reproductive performance of *S. serrata,* an understanding of these egg characteristics and the effects they have on each other must be gained.

Fecundity is one of the most important life-history parameters for any commercially farmed species and accurate estimates of fecundity are of great interest to hatchery managers (Stewart and Kennelly 1997). In many crustaceans, the eggs are physically attached to the pleopods of the female from the time of extrusion until hatching. The number of eggs a female can produce in each batch is limited by the available attachment space on the pleopods, which is determined by the size of the female and the size of the eggs (Stewart and Kennelly 1997). A positive relationship between fecundity and female size has been found for most palinurid lobsters (Phillips and Sastry 1980), the freshwater crayfish *Astacus leptodactylus* (Harlioğlu and Turkgulu 2000) and the velvet swimming crab *Necora puber* (Norman and Jones 1992). In decapod crustaceans, however, the relationship between female size and fecundity appears to be highly variable, with reports

of both intraspecific as well as annual variability (Corey 1987, Ennis 1991, Clarke *et al.* 1991).

Studies on the relationship between egg size and female size have yielded conflicting results. Mason (1978) showed a positive relationship between egg size and carapace width in the fresh water crayfish *Pacifastacus leniusculus*. Hines (1982) showed that egg size is positively related to female size in 21 species of brachyuran crabs. In a later study, however, Hines (1986) presents data showing that there is a positive relationship between egg size and female size only in the largest of brachyuran crabs, such as in the family Majidae (spider crabs).

Eggs of aquatic invertebrates increase their volume significantly during embryonic development by a slow and steady intake of water through osmosis (Davis 1968). This trend, though highly variable, has been observed by a number of authors working on decapod larval development (Davis 1964, Costlow 1965, Ling 1967, Wear 1967). Increase in egg volume during development varies greatly between species, with egg volume increasing by up to 54.4 % in *Megalobrachium soriatum* (Reid and Corey 1991), while egg volume in *Petrolisthes elongatus* (Jones 1977) increases by less than 15 % during embryonic development. Since space for egg attachment is limited on the pleopods of the female, it is expected that a loss of eggs through growth would occur as egg volume increases (Lardies and Wehrtmann 1996). This loss of eggs during development may therefore affect the validity of hatching success rate estimates. Hatch success rate is dependent on a variety of factors including parasites and diseases (Hansen *et al.* 1989), parental behaviour (Fernandez *et al.* 2000), increase in egg volume and loss of eggs (Lardies and Wehrtmann 1996).

Two of the most common problems in larval rearing of both marine fish and crustacea is the availability of a constant supply of eggs and larvae throughout the year and obtaining high levels of survival for each batch of larvae (Slavesen and Vadstein 1995). Amongst others, this requires a regular supply of disease and parasite free eggs. These criteria can be met largely through artificial incubation of eggs under controlled and sterile

environments. This is easily achieved in fish species where the eggs are separate and can be incubated under optimum water quality and environmental conditions. The eggs of *Scylla serrata,* however, are firmly attached to the pleopods by strong fibres. To obtain a high hatching rate therefore requires that the female crab be maintained under optimal water quality and environmental conditions.

The objectives of this study were to gain a better understanding of the following spawning and egg characteristics of *S. serrata* in captivity.

- Captive spawning patterns and synchronicity
- Effect of crab size on time taken to spawn
- Relationship between crab size and fecundity
- Relationship between egg size and crab size
- Incubation time
- Hatch success rates
- Embryonic development
- Viability of dropped eggs

Materials and methods

Each morning before cleaning, all crabs in the maturation system were examined for eggs. Females that had spawned were easily identifiable by their extended abdomens and the attached egg masses. Once identified, the crabs were removed using a hand net and placed into a 25 L plastic bucket, half full of water from the maturation system. The female was then weighed to the nearest gram before being placed into a glass observation tank. The purpose of first placing the females in the observation tank was two-fold. Firstly it allowed for easy handling for sampling of eggs and determination of egg colour, and secondly to disinfect the female prior to transferring her into an incubation tank. The total time that each female spent in the observation tank ranged from 12 - 24 hours. The observation tanks consisted of 110 L glass aquaria each connected to a biological filter (Figure 3.2), from which water was airlifted back into the tank. The filter medium consisted of small pebbles and a synthetic polyester sponge.

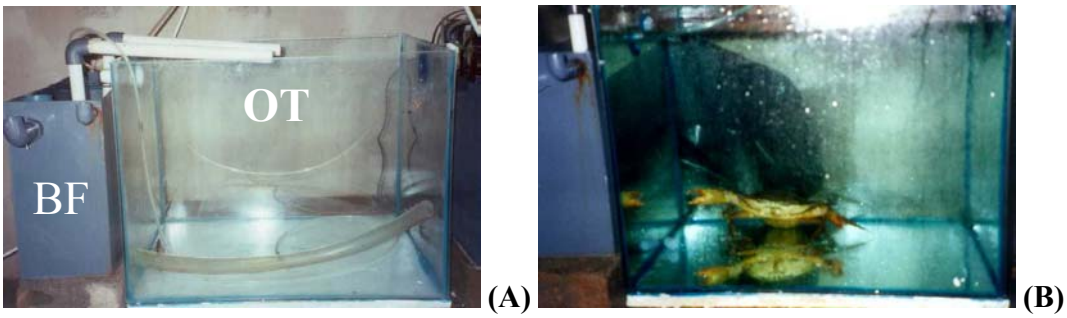


Figure 3.2: Observation tank (A) and biological filter (B). Observation tank is 600x400x450mm with a volume of 110 L. Biological filter has a volume of 20 L. BF = Biological filter, OT = Observation tank. Berried female in observation tank

Tank bottoms were cleaned daily by siphoning off any dropped eggs. All dropped eggs were weighed and examined under a microscope at 300 X magnification. If fungal or nematode infections (Figure 3.3) were detected on the eggs, the crabs were exposed to a 24 - hour bath of a 1 ppm malachite green and 10 ppm formaldehyde solution. During the disinfection period the circulation of water was stopped and aeration was increased. After 24 hours the water was slowly replaced with new filtered water (1μm) and the biological

filter reattached. Temperatures in the observation tanks ranged from 21 – 25°C in winter and 25 – 29°C in summer.

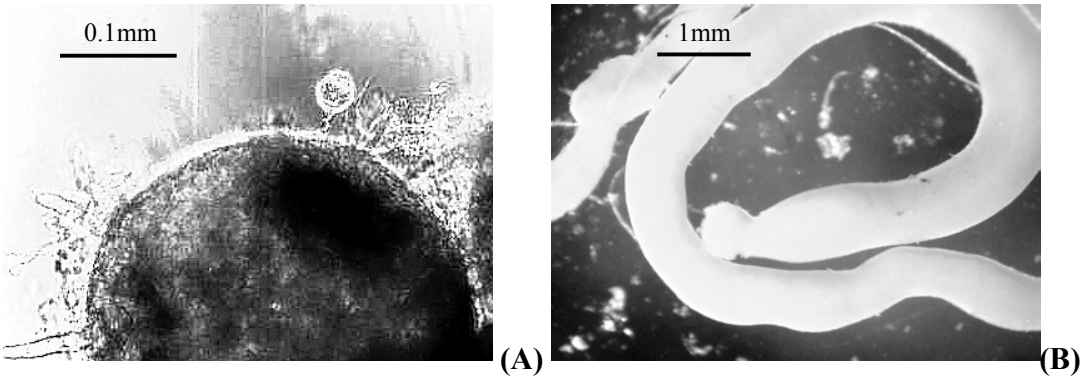


Figure 3.3: An egg infected with fungus *Lagenidium* spp. (A) and a nematode worm (B) found on the egg mass

Hatching occurred in the incubation tank. After all eggs had hatched the female was returned to the estuary from which it was captured. The incubation tank consisted of a 600 L black fiberglass container with a 30° conical bottom. A perforated false bottom was installed above the incline of the cone and this provided a perch for the berried crab. The perforations allowed any dropped eggs to fall through to the bottom of the tank, where they were removed daily by opening a tap. This ensured that the incubator remained clear of any debris, which might have acted as a surface for bacterial and fungal colonisation.

The incubation tank was connected to a 100 L biological filter and a UV sterilisation unit. Water flowed from the incubator through a 500 μm mesh sieve into the biological filter and was then pumped through the UV sterilisation unit before returning back to the incubation tank. A flow rate of approximately 200 L per hour was maintained at all times. All new water entering the incubation tank was first filtered to 1 μm and sterilised with ozone. Temperature was measured daily and an average temperature for each incubation period (n = 66) was calculated.

Spawning patterns in captivity

In order to establish if different sized crabs took longer to spawn in captivity, the time taken to spawn for each crab introduced into the maturation system was recorded. Time to spawn was then correlated to crab size and the time of year introduced into the maturation system. Spawning events between females were also recorded to establish if any degree of spawning synchronicity was present.

Fecundity

In order to estimate hatch success rate and the relative fecundities of different size females, fecundity had to be first estimated. Fecundity in this study was defined as the total number of eggs produced by a female during a single spawning event. Fecundity was calculated as follows. Crabs were weighed shortly after the extrusion of eggs, samples of eggs were removed using sterilised tweezers and weighed and counted. Three replicate samples of eggs were removed and counted per crab. To calculate total egg mass (TEM) the crabs were re-weighed after all eggs had hatched. To compensate for possible weight loss of the female during incubation, 25 non-berried crabs with carapace widths ranging between 90 – 175 mm were weighed, starved for twelve days (average incubation time) and then re-weighed. Weight loss was best described by the equation $y = -0.002x + 0.65$ ($r^2 = 0.52$; n = 25). Where y = log (% weight loss) and x = mass of crab after eggs hatched. To calculate weight loss during incubation, the mass after hatching was substituted into the equation above and the antilog taken. Total egg mass was then calculated as follows:

$$TEM = (ME - MH) + (MH \times Z)$$

where: TEM = total egg mass (g), ME = mass of crab at extrusion (g), MH = mass of crab after hatch (g) and Z = estimated weight loss during incubation (g)

Fecundity (F) was then calculated by extrapolating the number of eggs in a known mass into the total egg mass. Relative fecundity of the broodstock was calculated and expressed as the number of eggs per gram of female and was calculated as follows:

$$\text{Relative fecundity} = {No.\,of\ eggs}/{Wt.\,of\ crab\,(g)}$$

Relationship between individual egg size, mass and crab size

To determine whether egg size was dependent on crab size, triplicate samples of approximately 200 eggs were removed and placed onto blotting paper, to remove excess water, and then weighed on a digital balance (Excell 986) to the nearest 0.0001 g. All egg samples were taken at the time the newly berried female was placed into the observation tank for disinfection. Individual egg mass (IEM) for each replicate was estimated using the equation:

$$IEM = {M}/{NE}$$

where: M = mass of eggs and NE = number of eggs

Individual egg mass was then calculated as the average of the three replicates from each female.

In order to establish if egg diameter was dependent on broodstock size, a record of all newly extruded eggs was kept. Egg diameters were measured daily, from extrusion until hatching and the developmental changes in the egg recorded. Eggs were removed from the female as described above and kept moist to prevent dehydration. Egg diameter was measured using a compound microscope at 300 X magnification with a graduated eyepiece. Each day a sample of 30 eggs was measured.

Relationship between incubation period, egg diameter and embryonic development

From the 30 eggs examined each day for egg diameter an index of embryonic development was developed, which would aid in predicting the expected date of hatch. The following criteria were used to develop the index: egg size, egg colour, stages of embryonic development and movement within the egg.

It is a well-established fact that the development rates of eggs increase in proportion to temperature (Hines 1986). For every batch of eggs that was incubated (n = 58) temperature was measured daily throughout development. To establish the relationship between temperature and incubation period, the average water temperature was calculated and plotted against incubation period for each batch of eggs.

Relationship between hatch success rate and initial egg diameter, crab size, egg size at hatch, incubation period and time taken to spawn

In order to establish if hatch success rate was affected by any of the broodstock or egg parameters, the following steps were taken. On completion of the hatching process the female was removed from the incubation tank. The zoea were then evenly distributed in the water column by increasing the aeration level. Ten 50 ml sub samples were taken from the tank and the number of zoea in each counted. The number of viable larvae in the incubation tank was calculated using the equation:

$$NVL = \left[M \times \left(\frac{VT}{VS} \right) \right]$$

where: M = the mean number of zoea in the sub samples, VT = the volume of the tank and VS = the volume of the sub sample

Hatch success rate was then calculated using the equation:

$$\text{Hatch success} = \left(NVL / F \right) \times 100$$

where: NVL = number of viable zoea and F = fecundity

Hatch rate was then correlated with egg diameter, crab size, egg size at extrusion and hatch, incubation period and “time to spawn”. “Time to spawn” was the time between placing the females into the maturation system and extrusion of eggs.

Egg dropping

All dropped eggs were collected and examined under a microscope (300 X magnification). This was done to establish whether eggs were passively dropped due to a lack of attachment space on the pleopods as egg diameter increased or through the active grooming of egg mass by the female. Eggs removed from the observation and incubation tanks were weighed and measured daily. Eggs were also examined for fungal and nematode infestations. The percent of dropped eggs in each batch during incubation was then calculated by dividing the weight of dropped eggs into the calculated total egg mass (TEM). Dropped eggs were also examined to see if they were viable or dead. The proportions of dropped eggs were then correlated to crab size, incubation period, size of egg at extrusion and hatch rate. The proportion of dropped eggs from batches with fungal or nematode infestations were compared to batches showing no infestations.

Viability of in-vitro incubation

To establish if hatch rate could be improved by harvesting eggs from females, using an in-vitro incubation technique, nine experiments were conducted. Approximately 2 g of eggs were removed from the female just after extrusion. Before placing the eggs into the incubation vessels, the egg samples were gently pried apart as strands of eggs as much as possible ensuring that the eggs did not clump together. On average there were 600 strands of eggs incubated in each trial. The eggs were incubated in four 500 ml glass vessels that were placed in a water batch at 27±1^{o}C. Each incubation vessel was supplied with an air supply to ensure constant movement of the water around the eggs.

The eggs in each vessel were pipetted into a new clean incubation vessel daily, with water at the same temperature. This was done to minimise the chances of fungal infections and to ensure that salinity change, due to evaporation, was negligible. Samples of eggs, usually consisting of 2 - 3 strands of eggs (200 - 300 eggs), were removed daily from each incubation vessel and examined under the microscope for changes in development, measuring of egg diameter and examined for the presence of fungal infection. Hatch rates were calculated on the day of hatching by calculating the ratio of

empty egg cases to un-hatched eggs on the egg strands. To do this a sample of four egg strands consisting of 300 - 400 eggs were removed from each incubation vessel. Hatch rate was estimated as the average of the four replicates.

Data analysis

The objectives of this study were to gain an understanding of the spawning and egg characteristics of *Scylla serrata* in captivity. Thus a descriptive approach to data analysis was followed. All dependant variables were tested for normality using the Kolmogorov-Smirnov test at a 5% level of significance If the data were non-normally distributed they were either log or square root transformed. If the data were still non-normally distributed non-parametric tests were used.

Regression analysis and Spearman rank correlations were used to quantify and describe the relationships between variables in both the spawning and egg data sets. The null hypothesis was rejected at a P value ≤ 0.05 for all tests. Regression analysis was used to test whether the time taken for broodstock to spawn was dependent on crab size. Spearman rank correlation was used to identify the relationships between the time taken to spawn and crab size with the resulting egg variables.

Regression analysis was also used to establish the relationship between incubation period and hatch success. For the in-vitro incubation data a student t-test was used to establish if hatch rate of artificially incubated eggs was significantly different from that of eggs incubated by the female.

Results

Spawning patterns in captivity

Spawning by females took place approximately 38 ± 23 days after they had been placed in the maturation system (Figure 3.4).

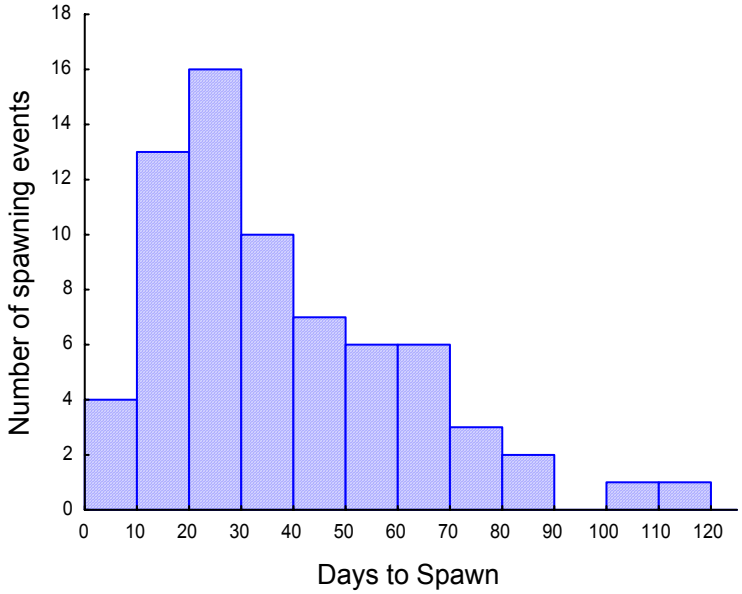


Figure 3.4: Number of days in captivity before females spawned (n = 69)

Spawning in the maturation system usually followed a trend, whereby spawning by one crab was invariably followed by spawning of other females within a 72-hour period. This "grouped" spawning would then be followed by a period during which no spawning events occurred. Of all spawning events, 61.8 % occurred in clusters of two to four crabs that spawned within 72 hours of each other (Table 3.1).

Table 3.1: Observed spawning patterns of female crabs in the maturation system over 23 months (n = 69)

Spawning patterns observed	% of total spawning events
1. Crabs spawning within 2-3 days of each other	61.8
2. Crabs spawning within 7 days of each other	28.7
3. Single spawning event separated from other spawning events by at least 10 days	9.4

In total, 64 % of the 112 females stocked into the maturation system spawned. During the course of the study, five females introduced into the system moulted. Three of the females were cannibalised while in the soft-shelled state, and the other two that did not spawn were released back into the estuary.

Although there was a peak in spawning activity in August, during which 40 % of the females in the maturation system spawned, spawning occurred throughout the year (Figure 3.5). There were no clear patterns in terms of the occurrence of spawning events and time of year with captive mud crabs.

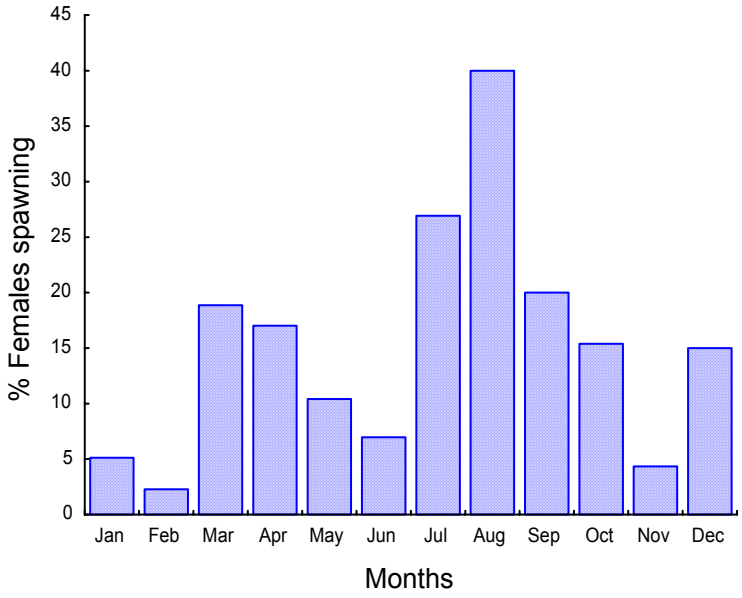


Figure 3.5: Percent of broodstock that spawned each month within the maturation system (n = 112)

There was also no statistically significant relationship between crab size (carapace width) and the time taken to spawn in the maturation system ($p > 0.05$) (Figure 3.6).

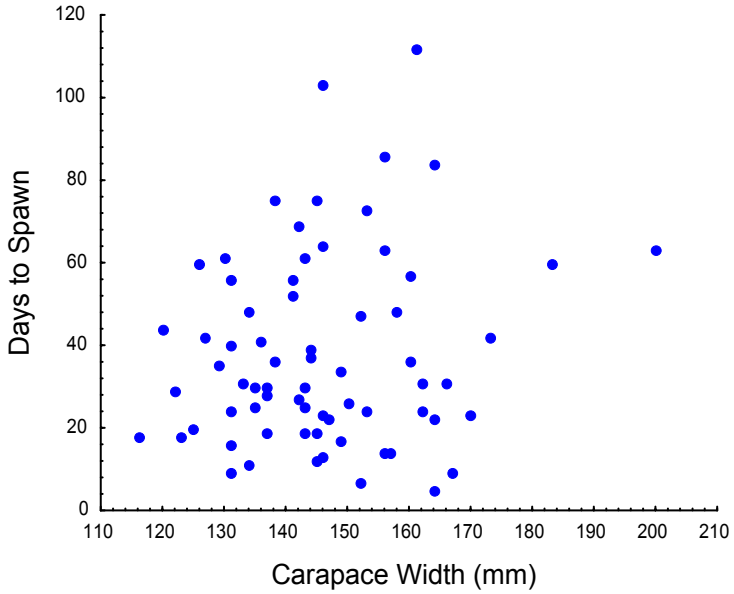


Figure 3.6: Crab size and time (days) to spawn in the maturation system (n = 69)

Fecundity

There was a significant (p = 0.026), but weak positive relationship between carapace width and total fecundity. Larger crabs had higher total fecundities (Figure 3.7).

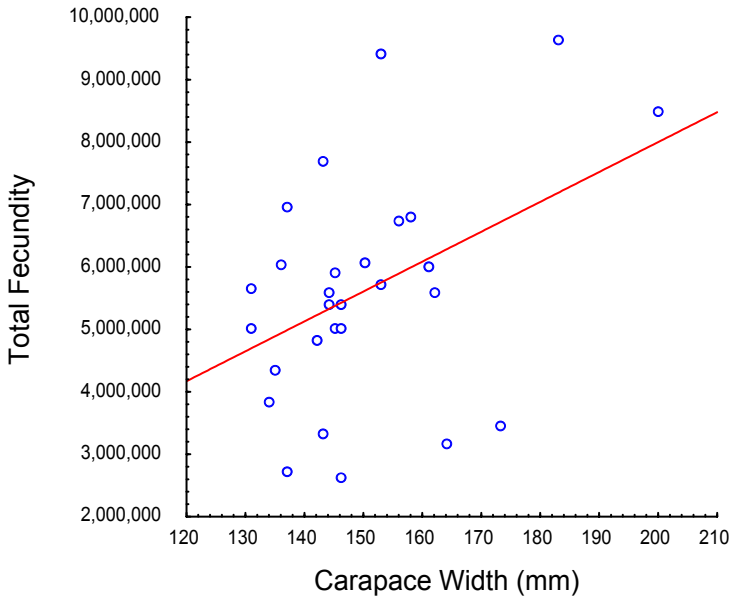


Figure 3.7: Relationship between crab size and total fecundity (r^2 = 0.176, p = 0.026, n = 28)

Crabs between 131 and 144 mm CW had an average total fecundity of 4.03 ± 1.17 million while crabs between 146 and 181 mm CW had and average total fecundity of

7.98 ± 1.79 million eggs. Relative fecundity was negatively correlated to crab size ($p = 0.004$) with larger crabs having lower relative fecundities (Figure 3.8).

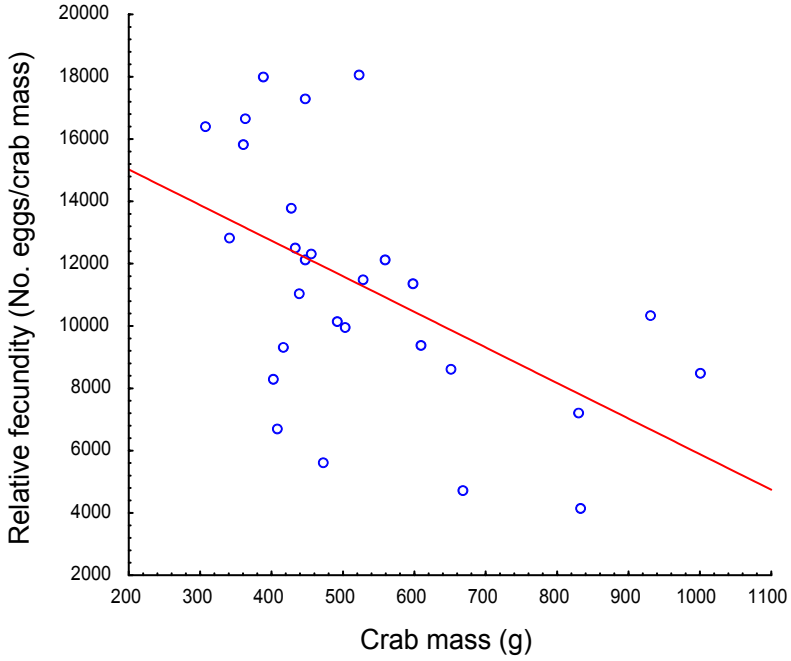


Figure 3.8: Relationship between crab size and relative fecundity ($r^2 = 0.274$, $p = 0.004$, $n = 28$)

Egg size and mass

Individual egg mass (IEM) ranged between 16.2 µg and 31.8 µg with an average mass of 22.4 µg. Individual egg mass showed little variation between different egg batches (Std.Dev = 5.1 µg). Individual egg mass was significantly correlated with crab size ($p = 0.012$) (Figure 3.9), but showed no relationship to any of the other parameters measured (Table 3.2).

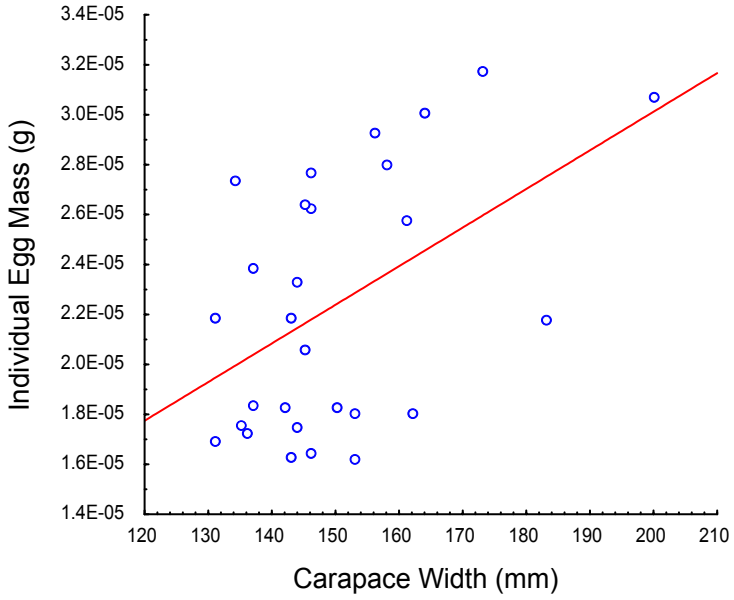


Figure 3.9: Relationship between individual egg mass and crab size ($r^2 = 0.231$, $p = 0.029$, $n = 28$)

Table 3.2: The correlation between individual egg mass and various egg and broodstock parameters. Significant correlations, (at a value of $p<0.05$), are indicated with an asterix ($n = 28$)

Individual egg mass Vs.	**p-value**
Days to spawn	0.086
Egg size at extrusion (mm)	0.424
Crab size CW (mm)	0.029*
Total fecundity	0.873
Hatch success (%)	0.670

Egg diameter at extrusion varied between crabs, ranging from 0.28 to 0.32 mm. Egg diameter was not correlated to crab size ($p \geq 0.05$) (Figure 3.10) or time spent in captivity ($p \geq 0.05$).

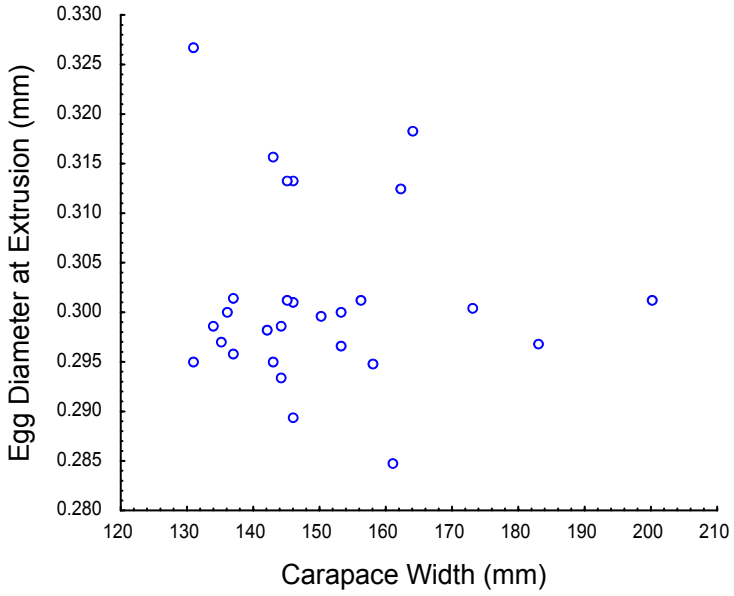


Figure 3.10: Relationship between egg diameter at extrusion and crab size (n = 28)

During incubation the increase in egg diameter is best represented by the polynomial equation $y = 0.299 + 0.002x + 0.001x^2$, where y = egg diameter and x = incubation period (days) ($p < 0.001$) (Figure 3.11). On average the egg diameters increased by 0.007±0.004 mm per day from extrusion until day 6. From day 6 onwards, daily increase in egg diameter accelerated to 0.012 ± 0.006 mm per day until hatching.

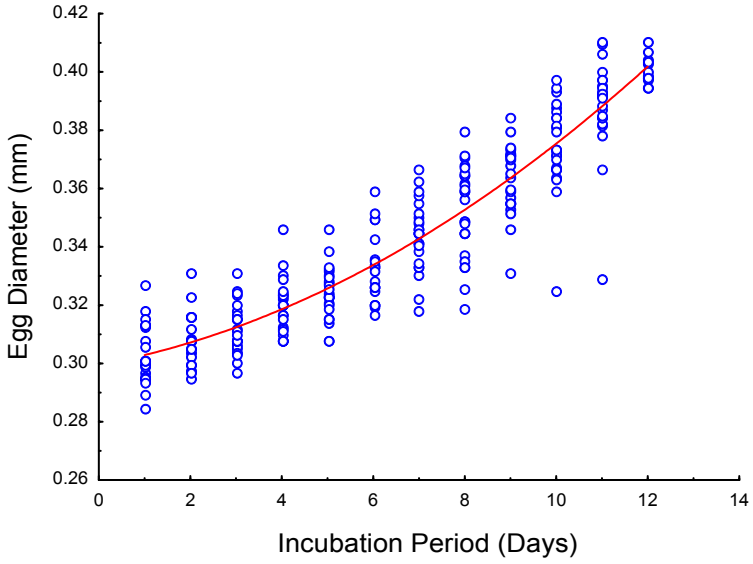


Figure 3.11: Daily increase of egg diameter during incubation. Average temperature during incubation was 27 ± 0.7 °C (n = 18)

Hatch rate

The average hatch rate in this study was 84 ± 6 %. There was a weak correlation between crab size ($p \leq 0.025$) and hatch success rate (Figure 3.12) with hatch success rate increasing with crab size. There was no relationship between hatch success rate and any of the other recorded parameters (Table 3.3).

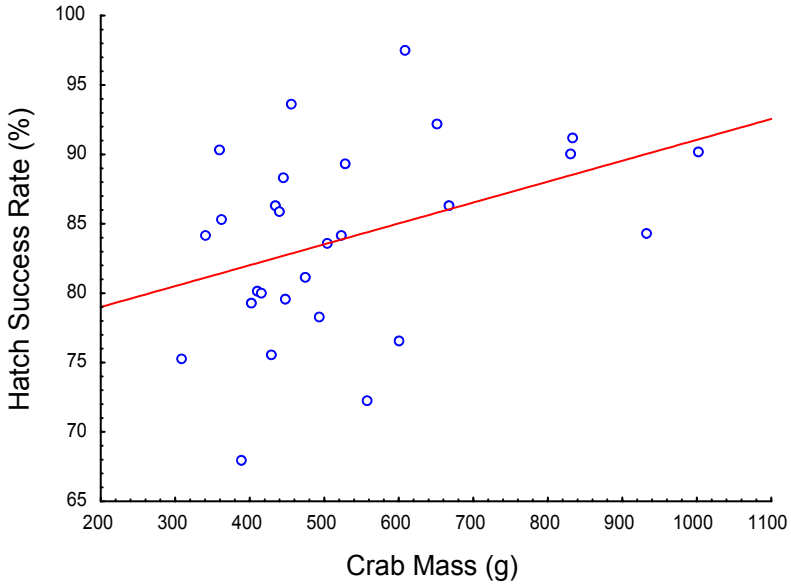


Figure 3.12: Relationship between hatch success rate and crab size ($r^2 = 0.154$, $p = 0.025$, $n = 28$)

Table 3.3: Relationship between hatch rate and various egg and brood stock parameters. Significant correlations ($p < 0.05$) are indicated with an asterix. $n = 28$

Hatch rate vs.	**p-value**
Time taken to spawn	0.131
Egg size at extrusion	0.920
Egg size at hatch	0.302
Incubation period	0.922
Crab mass	0.025*
Individual egg mass	0.788
Fecundity	0.942

Relationship between incubation period and temperature

The relationship between incubation period and temperature appears linear and is best represented by the equation, $IP = 44.54 - 1.2T$, where: IP = incubation period and T

= incubation temperature (Figure 3.13). Eggs incubated at 27.5 ^{o}C took ± 11.5 days to hatch while eggs incubated at 26 ^{o}C took ± 13.3 days to hatch

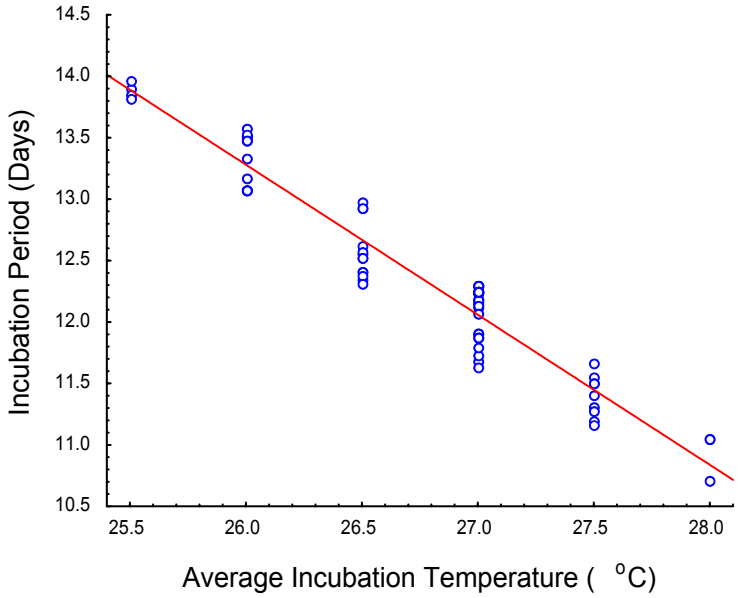


Figure 3.13: Relationship between incubation period and average incubation temperature ($r^2 = 0.94$, $p < 0.001$, $n = 58$)

Table 3.4: The minimum, maximum, mean and standard deviations of egg and broodstock parameters

Parameters	**n**	**Min**	**Max**	**Mean ± SD**
Egg diameter at extrusion (mm)	28	0.285	0.327	0.301±0.01
Egg diameter at hatch (mm)	28	0.382	0.414	0.398±0.006
Incubation temperature (^{o}C)	66	25.9	29.3	27.0±0.7
Incubation period (hours at 27^{o}C)	66	233	325	294±21
Days to spawn	69	5	112	38±23
Crab mass (g)	96	270	1000	488±144
Carapace width (mm)	96	116	200	145±15
Relative fecundity (number of eggs/g female)	28	3824	25502	10654±4068
Total fecundity (millions)	28	2.64	9.66	5.79±2.07
Individual egg mass (µg)	28	16.2	31.8	22.4±5.1
Hatch success (%)	28	68	97	84±6
Hatch synchronicity (Hours)	28	0.5	18	5±4

Ontogenetic development could be divided into six easily recognisable stages (Table 3.5), based on morphological characteristics of the egg and embryo. Using these stages it was possible to predict the expected date of hatch (within 1 - 2 days).

Table 3.5: Observed egg and embryo development stages from extrusion to hatching. Average incubation temperature 27±0.7 ^{o}C. Eggs were observed under a microscope (300X magnification)

Stage	Days after extrusion	Mean egg diameter ± SD (mm)	Characteristics
1	1	0.30±0.01	Yolk fills the entire egg; no division of the yolk has taken place.
2	4	0.32±0.01	Yolk has started to separate from the one side of the chorion. Yolk appears granular in appearance.
3	6	0.34±0.01	Eyespots are starting to develop, and are discernable as small red/brown smears.
4	8	0.36±0.01	Eyespots are clearly visible and the body shape of the zoea can be seen. Heartbeat is noticeable at this stage.
5	10	0.38±0.01	Pigmentation of the body and vascular system has become dark and clearly visible. Heartbeat is more pronounced and the body appendages can be easily recognised.
6	12	0.40±0.01	This stage is within 24 hours of hatch. The zoea are moving within the egg by contracting and flexing their appendages. Heartbeat has speeded up and is clearly visible even under low magnification (100X).

Observations on egg dropping

All female crabs dropped eggs during incubation. The weight of eggs dropped varied greatly and ranged from 0.7 % to 22 % of the total egg mass (mean = 6.5 %, Std.Dev. = 6 %). There was no relationship between the proportion of eggs dropped and crab size, total egg mass, egg size at extrusion or incubation period ($p > 0.05$) (Table 3.6). The proportion of eggs dropped appeared to be associated with the extent of grooming activities of the female, but this was not quantified.

Those batches of eggs that had fungal or nematode infections had a significantly higher ($H_{1, 28} = 14.1$, $p \leq 0.0002$) proportion of dropped eggs than "clean" batches of eggs and this obviously affected the hatch success rate (Table 3.7).

Table 3.6: Relationship between proportion of eggs dropped and the recorded brood stock and egg parameters. Significant correlations ($p < 0.05$) are indicated with an asterix

Dropped eggs Vs.	**p-value**
Fecundity	0.694
Incubation period	0.734
Size of egg at hatch	0.899
Hatch success rate	0.002*
Mass of crab	0.061

Table 3.7: Comparison of proportion of eggs dropped between infected egg batches (nematode and fungal infections) and uninfected egg batches.

	Infected batches (n = 9)		**Uninfected batches (n = 19)**	
	Mass of eggs dropped (g)	**% total egg mass**	**Mass of eggs dropped (g)**	**% total egg mass**
Minimum	6.2	4.3	0.7	0.6
Maximum	21.6	22	9.8	12
Mean±SD	15.1±4.4	13.4±5.8	3.7±2.6	3.5±3

In-vitro incubation

Nine *in-vitro* incubation trials were attempted. The hatch rate in the in-vitro experiments were significantly lower ($p \leq 0.0001$) than that of eggs incubated by the female. On average only 25 ± 5.4 % of the eggs hatched successfully in the in-vitro trials in comparison to the mean natural hatch rate of 83 ± 6.1 %. Fungal and parasitic infections occurred in all in-vitro trials. This was manifested by the high egg mortality, from which it was concluded that in-vitro incubation of eggs, as an alternative to natural incubation is not a viable option for *S. serrata.*

Discussion

Spawning patterns in captivity

Maturation and spawning of *Scylla* species, both in the wild and captivity, has been observed to occur all year-round (Mann *et al.* 1999, Le Vay 2001). Seasonal peaks in spawning activity do occur but differ according to climate and latitude (Heasman *et al.* 1985, Quinn and Kojis 1987, Robertson and Kruger 1994, Mann *et al.* 1999). In this study, the peak spawning period in captivity occurred in winter (July-August). This differs from other studies conducted on natural populations of *Scylla serrata* in South Africa. Hill (1974, 1975) found that mature females extrude their eggs summer (February – March) in the St. Lucia system, while Robertson and Kruger (1994) recorded that spawning in the Mlalazi Estuary and Richards Bay occurs throughout the year, with a peak in reproductive activity from November to April.

The discrepancy between peaks in spawning activity observed in the maturation system and those in the wild may be a result of captive conditions, in which water temperature and food availability remained high throughout the year. In Australia, Mann *et al.* (1999) found that crabs spawn all year round in captivity, while natural populations showed distinct seasonal spawning activity. Heasman *et al.* (1985), working on a wild population of S. *serrata* in Moreton Bay (Australia), found that there was no loss in the condition of ovaries during the colder months when little or no spawning occurred. This may explain why, when conditions become favourable, there is some degree of synchronous spawning in the wild. He suggests that the ability to retain ovary condition may be one of the factors that allow spawning activity in captivity to continue throughout the year, irrespective of when the crabs were caught.

During this study, spawning events were often grouped together, with a few females spawning within a short period of each other, followed by extended periods where no spawning took place. Although this phenomenon has hitherto not been documented for *Scylla serrata* in captivity, it has been observed (Colin Shelly, Department of Primary Industries, Australia Queensland, *pers comm*). Spawning synchronicity appears to be common amongst crustaceans (Olive 1992) and is often associated with food abundance.

Sex pheromones are also known to play a role in the sexual behaviour of decapod crustaceans, particularly during the moulting cycle of mature females (Gleeson 1991, Bamber and Naylor 1996). There is also some evidence to suggest that hatching eggs release hormones, which could induce other females to spawn (Forward *et al.* 1987). Another explanation for synchronous spawning may be that wild crabs mature their ovaries with some degree of synchrony. In this study, there was no relationship between those crabs that spawned in short succession of each other, neither in terms of size nor the time they spent in captivity.

Fecundity

In several crustaceans there is a linear relationship between the number of eggs per brood and the size of the female. This has also been observed for the freshwater prawn *Macrobrachium lamarrei* (Shakuntala 1977), the freshwater crayfish *Astacus leptodactylus* (Köksal 1988), the Balmain bug *Ibacus peronii* (Stewart and Kennelly 1997), the crayfish *Procambarus (Austrocambarus) ilamasi* (Rodriguez *et al*. 2000) and the velvet swimming crab *Necora puber* (Norman and Jones 1992). Prasad and Neelakatan (1989) showed a similar direct relationship between size and fecundity in *S.serrata* up to a size of 140 mm carapace width. During the present study, a significant correlation ($p < 0.05$) was found between crab size and fecundity, with larger crabs having higher fecundities. The total fecundity and relative fecundity values obtained in this study were comparable to that recorded in Australia (Mann and Parlato 1995) and the Philippines (Millamena and Bangcaya 2001).

Despite the significant ($p = 0.026$) relationship between crab size and fecundity in this study, results were highly variable ($r^2 = 0.176$). Mud crabs are known to be able to fertilize more than one batch of eggs from a single mating (Ong 1966, Heasman *et al*. 1985) and it has been found that fecundity decreases with each successive batch of eggs produced (Ong 1966). As females were only kept for one spawning in this study, the smaller egg batches produced by some of the larger crabs may have been the second or

third batch of eggs from a single mating, while large batches of eggs from smaller crabs may have been the first spawning. This can only be tested using captive broodstock where time of moulting and mating is known and the subsequent spawning events and batch sizes are recorded.

In a review by Hines (1991) on the fecundity and reproductive output of nine species of brachyuran crabs, egg size did not vary significantly within species and mean egg size was not correlated with female body size. The same trend was observed during this study, where egg diameter was not related in any way to female size, time of year extruded, temperature or the time the females spent in captivity. Egg diameters increased at a relatively steady pace throughout ontogeny. The rate of increase in egg diameter was, however, dependent on temperature. Eggs incubated at higher temperatures hatched at the same size but in a shorter period. There was an accelerated increase in egg diameter from the time the embryonic heartbeat was first observed (after 7-8 days). This is similar to the observations made by Wear (1974) for several decapod species e.g. the green crab (*Carcinus maenas*), the nut crab (*Ebalia turberosa*) and some lobsters (*Galathea dispersa, Galathea squamifera*).

Incubation period and temperature

In most crustaceans the incubation period is highly dependent on temperature (Heasman and Fielder 1983, Hines 1986). Incubation periods of the Moreton Bay (Australia) population of *S. serrata* in early spring, at water temperatures of 18 – 20 ^{o}C, were twice as long as those during the summer when water temperature ranged between 26 – 28 ^{o}C (Heasman and Fielder 1983). Water temperatures in estuaries and coastal waters along the north coast of South Africa range between 17 – 22 ^{o}C in winter and 23 – 30 ^{o}C in summer (Robertson and Kruger 1994). In this study, it was shown that the incubation period ranged from 333 hrs at a mean temperature of 25.8 ^{o}C (range 25.6 – 25.9 ^{o}C) to 262 hrs at a mean temperature of 28.1 ^{o}C (range 28.0 – 28.3 ^{o}C). By plotting published values (Figure 3.14) it would appear that there is an exponential relationship between temperature and incubation period for *S.serrata* between the temperatures of 21 – 29 ^{o}C.

This relationship is best represented by the equation, $y = 6029.7 - 406.9x + 7.2x^2$ where: y = incubation period and x = temperature.

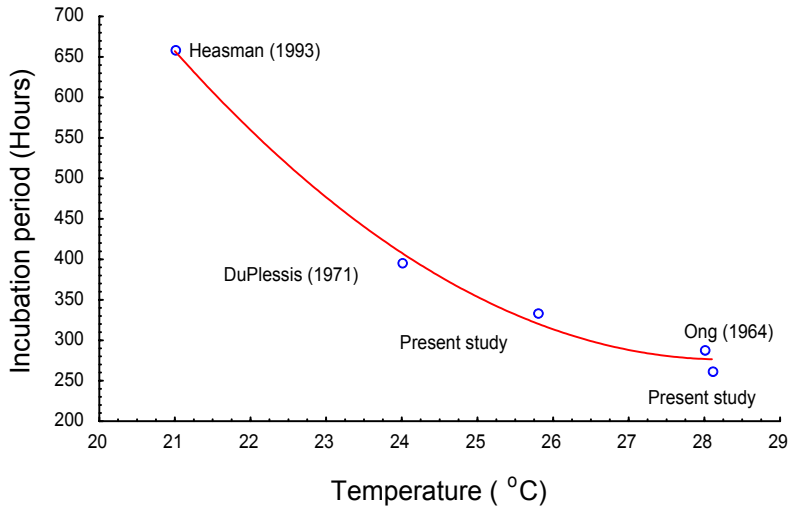


Figure 3.14: Relationship between incubation period and temperature for *S. serrata* ($r^2 = 0.90$, $p = 0.01$).

Heasman and Fielder (1983) found that *S. serrata* females aborted all eggs at temperatures lower than 17 °C, and this was accompanied by severe fungal and ciliate infections. In this study incubation temperatures seldom dropped below 25 °C, but in two cases where the temperature dropped below 23 °C, the eggs were highly infected with fungi and nematode worms and had to be discarded.

Hatch success rate

Throughout the study, hatch rates remained high (Table 3.4) and appeared to be independent of crab size or the time the females spent in captivity. As mentioned previously, low total fecundities in some large crabs may be a result of females spawning their second or third batch of eggs. However, there was no significant difference between hatch success rate and the size of the egg mass. This is in contradiction to findings by Ong (1966), where successive spawning resulted in lower fecundities, paler eggs and poor hatch rates.

Developmental stages

The results presented in this study have shown that it is possible to develop an index of development, which could be used to predict the time (day) of hatching. This type of staging index has been used on a variety of species to estimate fertilization rates as well as to predict date of hatch. By using the staging index developed in this study, time/day of hatching could be established fairly accurately to within 24 hours. The predictive capabilities of this index allowed for the timely preparation of live foods and rearing systems for the zoea.

Egg dropping

All females dropped some eggs during the incubation period. Numerous reasons have been identified that may contribute to egg loss, e.g. abrasion of the egg mass against the substrate, increasing egg volume resulting in a shortage of space for attachment on the female abdomen (Lardies and Wehrtmann 1996), weak attachment to the pleopods (Mathews and Reynolds 1995), ciliate infestations (Quinito *et al.* 2001) and predation (Shields *et al*. 1990). During this study, egg loss during incubation generally remained below 5 % of the total egg mass, although in those batches that were infected with fungi or nematodes, egg loss increased to 22 % of the total egg mass. No relationship could be found between incubation temperature and egg dropping or the presence of fugal or nematode infestations.

If eggs are dropped but are still viable (as shown by the in-vitro incubation of dropped eggs), then the causes of egg dropping must be similar to those suggested by Lardies and Wehrtmann (1996) and Mathews and Reynolds (1995). Throughout the study, grooming of the egg mass by the females was observed. The females did this by lifting their abdomens off the substratum and picking at the egg mass with their second and third walking legs. Through inspection of the egg masses it appears that grooming occurred more often in batches that had fungal and nematode infestations. The higher proportion of dropped eggs in infected egg batches may therefore be as a result of increased grooming activities by the female. Egg dropping also decreased significantly in infected batches of eggs after exposure to formalin and malachite green.

In-vitro incubation

Several studies have been undertaken to test the viability of in-vitro incubation of crustacean eggs as an alternative to natural incubation (King 1993, Perez *et al.* 1999, De Caluwé *et al.* 1995_b). Theoretically, in-vitro incubation allows for control of ambient conditions such as water quality and diseases. In-vitro incubation of crustacean eggs has been achieved in species such as the white-clawed crayfish (*Austropotamobius pallipes*) (Perez *et al.* 1999) and *Macrobrachium rosenbergii* (De Caluwé et al. 1995_b). In both of the above-mentioned species, eggs were removed from the females before in-vitro incubation. In this study, the eggs were firmly attached to the fibres on the pleopods and could not be removed without damaging them. It was for that reason that egg strands were incubated after they had been cut from the egg mass. However, even when strands were incubated, the strands tended to get tangled as they circulated in the incubation vessels, causing the eggs to clump together. This clumping may have reduced the surface area available for adequate oxygen transmission to the embryos, resulting in a lower hatching success rate.

The oxygen requirements of developing eggs increases as development progresses (Naylor *et al.* 1999) and is the reason why ovigerous females physically aerate their egg masses during the later stages of development (De Caluwé *et al.* 1995, Naylor *et al.* 1999). During this study, all ovigerous females aerated their egg masses and, at time of hatch, actively flexed and contracted their abdomens expelling newly hatched zoea. This movement is thought to aid the hatching process by assisting the hatching zoea break free from the egg case (Perez *et al*. 1999). In most artificial incubation studies, mortalities have been particularly high in the final stages of development just prior to hatching (Carral *et al*. 1992, Perez *et al*. 1999). During this study, development of all batches of in-vitro incubated eggs proceeded as normal but only 25 % of the zoea hatched successfully. This may be as a result of the lack of natural stimuli to aid the zoea to break free of the egg case.

Conclusions

Spawning of *Scylla serrata* in captivity occurred year round in northern KwaZulu-Natal in South Africa. All wild caught mature females were capable of spawning and acclimated readily to captivity. On average, spawning occurred within 38 days in captivity. Time to spawn in captivity, egg size and hatch success were independent of crab size. Fecundity was related to female size, although highly variable. Incubation time was highly correlated to incubation temperature. At 27^{o}C hatching takes place within 292 ± 12 hours after extrusion. Fungal and nematode infestations can be controlled effectively using a 1ppm malachite green/10 ppm formalin cocktail administered overnight. The egg development stage index, developed in this study, proved to be highly effective in predicting the date of hatch, allowing sufficient time to prepare the larval rearing system and to produce adequate quantities of live food. Egg dropping occurred in all batches of eggs and was significantly influenced by the occurrence of fungal and nematode infestations of the egg mass. The amount of eggs dropped was, however, independent of temperature and incubation period. In-vitro incubation of *Scylla serrata* eggs proved to be less successful than in-vivo incubation of the eggs by the female. In-vitro incubated eggs were prone to fungal infections and nematode infestations and tended to clump together, restricting the amount of water circulation around the embryos. Hatch rates of in-vitro incubated eggs were significantly lower than in-vivo incubated eggs, despite normal embryonic development.

Chapter 4

Evaluation of egg quality

Introduction

Success in any crustacean aquaculture venture depends, among other factors, on the quality and health of the larvae and post larvae used for grow-out (Dhert *et al.* 1992). Assessment of the health and fitness of crustacean larvae has become of increasing importance, particularly for the shrimp and prawn farming industries where post larval quality can significantly affect final pond yields (Tayamen and Brown 1999). Increasingly, producers and buyers are becoming aware of the importance of larval quality and are applying visual criteria such as colour, muscle development and activity levels to evaluate larval condition (Tackaert *et al.* 1989, Villalon 1991, Browdy 1992). However, these visual assessments are highly subjective and do not allow for the quantification of differences in quality. It is for this reason that visual assessments are now used in conjunction with stress tests to evaluate larval quality (Tackaert *et al.* 1989, Bauman and Jamandre 1990, Bauman and Scura 1990, Samocha *et al.* 1993, Samocha *et al.* 1998).

Research into captive spawning and rearing of *Scylla serrata* has identified several egg characteristics, viz. egg colour and hatch synchronicity, which affects larval quality (Ong 1966, DuPlessis 1971, David Mann, Bribie Island Aquaculture Research Centre, Queensland, Australia. *pers. comm*). Hatching synchronicity refers to the time taken from the commencement of the hatching process of a batch of eggs to completion. If batches of eggs take longer than 1 hour to hatch (from the beginning to the end of the process) they are considered inferior and the larvae discarded (David Mann, Bribie Island Aquaculture Research Centre, Queensland, Australia. *pers. comm*). Similarly, researchers often discard batches of pale yellow eggs in favour of darker, red batches of eggs (Ong 1966, DuPlessis, 1971).

Crustacean egg colour is determined mainly by carotenoid content of the yolk, and in particular astaxanthin (Harrison 1990). Carotenoids cannot be synthesised by crustaceans and have to be obtained from dietary sources (Harrison 1990). The accumulation of carotenoids in the ovaries during sexual maturation has led many researchers to speculate on their role in reproduction and larval development (Herring 1968, Gilchrist and Lee 1972, Gilchrist and Zagalsky 1983). Only recently, however, has a positive relationship between carotenoid content and spawning, fecundity and larval fitness been found in *Penaeus monodon* (Pangantihon-Kuhlmann *et al.* 1998) and the sea urchin *Lytechinus variegatus* (George *et al.* 2001, Pathirana *et al.* 2003). Similarly, carotenoid content in the ovaries and eggs of fish has also been shown to be positively related to egg quality (Miki *et al.* 1984, Watanabe *et al.* 1991, Bromage and Roberts 1995).

The biochemical composition of a "healthy" egg reflects the embryonic demands for both nutrition and growth (Harrison 1990). Given that crustacean eggs appear to be impermeable to organic substances (Claybrook 1983), due to the presence of chitin in the external membrane (Goudeau *et al.* 1987), suggests that the nutritional requirements of the developing embryo are contained entirely within the egg (Harrison 1990). In some crustaceans, e.g. penaeids, development is rapid with fertilized eggs hatching into nauplii within 24 hours (Primavera and Posadus 1981). These nauplii, however, still receive nutrients from the remaining yolk, which sustains them through several moults and metamorphosis into protozoea larvae (Harrison 1990). In other species, e.g. lobsters, development is protracted and may last as long as 9-12 months (Watt and Arthur 1996). In these cases the nutritional and energy reserves within the egg are even more critical as the embryos hatch into free-swimming larvae with no yolk reserves and are immediately dependent on exogenous nutrient sources (Sasaki *et al.* 1986, Harrison 1990).

Newly hatched *S. serrata* larvae have no yolk sac or yolk reserves to provide endogenous nutrition post-hatching; implying that larval viability is dependent on yolk quantity and quality. As a consequence, newly hatched larvae must have immediate access to an abundant and nutritionally adequate live food source. Analysis of the biochemical composition of the egg may, therefore, possibly serve as an indicator of egg quality.

Lipids, in particular, play an important role in the metabolism and reproduction of decapod crustaceans (Harrison 1990), as well as maintaining the functional integrity of biomembranes (Roustaian *et al.* 1999). Neutral lipids, particularly triglycerides (TG), are the major energy source in the egg and pre-feeding larvae (Teshima and Kanazawa 1983). These neutral lipids are comprised mainly of the non-essential fatty acids (NEFA) such as the saturated fatty acids up to 20-22 carbons. The most common NEFA are 16:0 (Palmitic acid) and the omega-9 family fatty acids.

Certain components of the yolk have been termed "essential" for the organism, as they can not be synthesised *de novo* (Sargent 1995, Morehead *et al.* 2001), and must be present in certain amounts to satisfy biological demands. The essential fatty acids (EFA) (i.e. those that cannot be synthesised *de novo*) are mainly comprised of polyunsaturated fatty acids (PUFA) such as 18:3(n-3) (linolenic acid) and highly unsaturated fatty acids (HUFA) such as 20:4(n-6) (eicosatetraenoic acid); 20:5(n-3) (eicosapentaenoic acid) and 22:6(n-3) (docosahexaenoic acid) (Halver 1989, McVey 1993, D'Abramo 1997).

The fatty acid composition of fish eggs, and in particular the EPA content, has been shown to be an important and valuable indicator of egg quality (Bromage and Roberts 1995). To evaluate whether this could also be used as an indicator of egg quality in *S.serrata,* the fatty acid composition of newly extruded eggs and newly hatched zoea were analysed. Egg composition as an indicator of egg quality can be assessed both directly and indirectly. Direct assessment would, for example, involve the comparison of the EFA composition of the eggs with egg colour, hatch success rate, hatch synchronicity, broodstock size and time spent in captivity prior to spawning. Indirectly egg quality may be assessed, for example, by correlating survival of newly hatched larvae in the stress tests to parameters such as egg colour, egg size, hatch synchronicity of different coloured egg batches and broodstock size. All experiments undertaken in this study were restricted to newly hatched first stage zoea. This was done purposefully as any feeding (irrespective of consistency) may mask the quality of the fertilized egg and the first zoeal stage.

Since the publication of Watanabe's (1983) paper, stress tests have been used to evaluate larval quality in many species (Dhert *et al.* 1992). Stress can be defined as "the reaction of an organism by a disturbed physio-biological balance to an abnormal impact of the environment" (Dhert *et al.* 1992). Stress tests are useful tools in that they produce quantifiable data on larval quality in a relatively short period (usually less than 96 hours). Stress tests deal with the dose-response relationship of a stressor and involve quantifying the toxic effect and correlating it to exposure. The simplest measurement of toxicity is lethality and is commonly termed the LD_{50}, which represents the concentration of a stressor at which 50% of the test organisms die within a given time (Timbrell 2000). Stress tests and visual assessments are now widely used to predict and identify quality in crustacean post larvae and juveniles (Castille and Addison 1986, Dhert *et al.* 1992, Tayamen and Brown 1999). In this study stress tests were undertaken on newly hatched crab larvae to retrospectively assess egg quality, which to our knowledge has not previously been attempted.

The most commonly used stressors are ammonia (Lin *et al.* 1993, Ostrensky and Wilson 1995, Chen and Chen 2000), starvation, formalin (Samocha *et al.* 1998) and salinity (Rees *et al.* 1994). Un-ionized ammonia diffuses across cell membranes in the direction favoured by its pressure gradient (Fromm and Gillette 1968). Increasing ammonia levels in the water results in a decrease in ammonia excretion and an increase of ammonia levels in the blood and tissue (Cavalli *et al.* 2000). The increase in ammonia in the blood leads to elevated blood pH, adverse membrane stability and enzyme-catalysed reactions, which may lead to death (Tomasso 1994). Work on *Macrobrachium rosenbergii* larvae has shown that the use of ammonia in a static 24-hour acute ammonia toxicity stress test was sensitive enough to distinguish between larvae fed on different diets (Cavalli *et al.* 2000).

Acute salinity stress tests have been used to distinguish between healthy and weak *Penaeus monodon* post larvae (Baybay 1989, Tackaert *et al.* 1989, Samocha *et al.* 1998) and are now commonly used in commercial hatcheries to evaluate post larval quality. Tolerance to osmotic or chemical stress is, however, variable between species

(Charmantier *et al.* 1988) and dependent on the age and nutritional status of the PL (Arellano 1990, Gomez *et al.* 1991, Rees *et al.* 1994). *Scylla serrata* zoea hatch at sea, where they are not exposed to significant fluctuations in salinity throughout their larval stages. Studies on the salinity tolerance of *S.serrata* zoea in South Africa have shown that 100% mortality occurs at a salinity of 17.5‰ (Hill 1974). It was mainly for this reason that salinity was chosen as one of the stressors.

Formalin is one of several chemicals used to control disease in crustacean larviculture and has been used to treat ciliates in *Callinectes sapidus* (Sindermann 1977), cnidarians in decapod crustacean larvae (Sandifer 1974), suctorian protozoans on *Macrobrachium* (Goodwin and Hanson 1975), and shell disease in crabs (Tareen 1982), amongst others. Since formalin was used in this study for disinfection and the treatment of eggs and zoea against ciliate and nematode infections, it was chosen as one of the stressors.

Crustacean embryos and pre-feeding larvae are licithotropic. The quality of newly hatched larvae is therefore determined by the biochemical composition of the yolk and its genetic make-up (Bromage and Roberts 1995). For this reason starvation was selected as another stressor to establish if newly hatched zoea from batches of eggs with different characteristics (e.g. colour and size) or obtained from small or large broodstock females showed differences in quality. The starvation test results were also used as a reference point against which to compare the performance of the first stage larvae in the other stress tests.

The aims of this study were to test the hypotheses that (1) egg colour can be used as a simple indicator of quality and (2) the fatty acid composition of the egg can be used as an indicator of egg quality. The first hypothesis was tested by seeking statistical relationships between egg colour and the following egg and broodstock parameters; broodstock size, the time the females spent in captivity prior to spawning, egg size at extrusion, hatch success rate and hatch synchronicity. The second hypothesis was tested by firstly seeking a relationship between the fatty acid composition of the eggs and their colour and then by seeking relationships between broodstock size, the time females spent

in captivity prior to spawning, egg size at extrusion, hatch success rate and hatch synchronicity.

In addition, stress tests were used in a retrospective manner to establish if any of the recorded broodstock, egg and biochemical parameters were reliable indicators of egg quality. This was done by correlating the LD_{50} values obtained in the respective stress tests to the time females spent in captivity prior to spawning, female size, egg size at extrusion, egg colour, hatch success rate, hatch synchronicity, biochemical composition of newly extruded eggs and the biochemical composition of newly hatched zoea.

Materials and methods

Evaluation of egg colour

Within 12 hours of spawning, berried females were removed from the maturation system, weighed and placed into the observation tanks (see Chapter 3). Four observers were asked to independently rank the egg colour of each batch of eggs (n = 28) from pale yellow to orange-red, on a points scale (1 = pale yellow to 5 = orange-red). Each egg bolus was then photographed (Agfa 100 ASA) in order to keep a record. In total 28 batches of eggs were photographed. Photographs were taken indoors under the same light conditions and at the same place. Three batches of different colour eggs are illustrated in Figure 4.1. All egg batches were incubated successfully, whereafter egg colour was correlated to hatch synchronicity, hatch success rate, female size and the time the female spent in captivity prior to spawning.

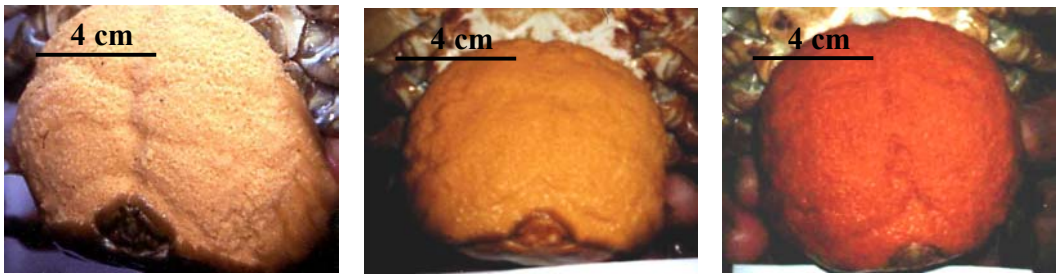


Figure 4.1: Range of egg colours: pale yellow (left) to orange (centre) and orange-red (right).

Hatch synchronicity and hatch success rate

Hatching generally occurred during the day, starting at about 09:00 to 10:00. To estimate hatch synchronicity the female in the incubation tank was observed once every half hour on the predicted hatch date (see Chapter 3) until hatching commenced. When hatching commenced the female exhibited rhythmic contractions of the abdomen, which stopped when all the eggs had hatched. The time between the beginning and end of this behaviour was recorded as the time taken for the eggs to hatch. Hatch success rate was calculated as described in Chapter 3.

Fatty acid analysis

Total lipid content, lipid class composition and fatty acid methyl ester content was determined and calculated for the broodstock diet, newly extruded eggs and newly hatched stage 1 zoea. Due to cost constraints only one sample of each of the feeds was analysed. Samples of newly extruded eggs (n = 28 samples) and newly hatched zoea (n = 16 samples) were collected between June 2000 and December 2001. Due to the random colour of different egg batches of the 28 egg samples analyzed, four batches were pale yellow, five were yellow, thirteen were orange yellow, four were orange and two were orange red in colour. Of the 16 zoea samples analysed one sample was from pale-yellow eggs, two samples were from yellow eggs, nine samples were from orange yellow eggs and four samples originated from orange coloured eggs.

The egg and zoea samples were prepared by placing them on blotting paper to remove excess water, homogenised and frozen to -5^{o}C before being freeze-dried. The freeze-dried samples were then placed into sealed glass vials and sent to the Artemia Reference Centre, University of Ghent in Belgium for analysis. Fatty acid composition was determined by gas chromatography. Fatty Acid Methyl Ester analysis (FAME) was conducted using a modified procedure of Lepage and Roy (1984). This method uses direct acid catalyzed trans-esterification, without prior extraction of total fat, on dry sample amounts ranging from 10 to 150 mg. Ten percent of an internal standard 20:2 (n-6) was added prior to the reaction.

Statistical techniques (See page 57) were used to test for any relationships between the fatty acid composition of eggs and larvae and egg colour, broodstock size, time spent in captivity prior to spawning, egg size at extrusion, hatch success rate and hatch synchronicity.

Stress tests

After the eggs had hatched, the female crab was removed from the incubation tank, using a scoop net, and the aeration within the tank turned off. This allowed any debris such as egg cases and dead zoea to settle to the bottom of the incubation tank, while the photo-

tactic zoea remained active at the surface. Zoea were then scooped out of the incubation tank near the surface of the water column using a 1 L vessel. Ten zoea were then randomly picked and individually pipetted into each of 3 replicate test vessels. All stress tests were conducted within 6 hours of hatching. The test vessels consisted of a 250 ml glass beaker, which was filled with 200 ml of the desired stressor. During all tests the larvae were not fed, no aeration was provided and temperature was maintained at between 27 – 29^{o}C. To reduce evaporation, each test vessel was covered with an opaque PVC lid for the duration of the test.

The stress tests used in this study followed a similar design to those conducted by Lin *et al.* 1993 and Ostrensky and Wilson 1995, where the test organisms were exposed to various concentrations of a given stressor and the percent mortality recorded after a given time period. Another method is to assess mortality at regular time intervals (as often as every 5 mins) throughout the duration of the test (Rees *et al.* 1994, Chen and Chen 2000). Although the latter method is generally regarded to be more sensitive, it is not practical when more than one stress test are running concurrently. Percent mortality was assessed after 24 hours in the formalin, ammonia and salinity tests, while in the starvation stress tests mortality was checked every 24 hours for 144 hours. Mortality was assessed by gently pippetting the zoea on the bottom of the test vessels into a petri dish where they were examined. Zoea were declared dead if there was no movement of the appendages and if the larvae did not respond when prodded with a glass pipette.

Prior to conducting the stress tests, range finding tests were conducted on three batches of zoea using a wide range of concentrations of each stressor. Each concentration tested in the range finding tests was conducted in triplicate. For the ammonia range finding tests concentrations of total ammonia (NH_3 and NH_4^+) ranged from 25 ppm to 450 ppm at 25 ppm intervals. The concentrations used in the salinity range finding test ranged from 35 ppt to 60 ppt at 5 ppt intervals and the formalin concentrations tested ranged between 2.5 ppm and 75 ppm at 12.5 ppm intervals. The upper concentration limit chosen for the

actual stress tests was the first concentration at which 100% mortality was recorded in the three replicate batches of zoea after 24 hours.

The salinity stress test consisted of four hyper-saline solutions and a control. The hyper saline solutions were made up by mixing uniodized sea salt in natural seawater. The four concentrations tested were 40, 45, 50, 55 ppt, respectively and a seawater control of 32-34 ppt.

For the ammonia stress tests a 1 000 ppm stock solution was made up by adding ammonium chloride to fresh seawater. This stock solution was then diluted to eight desired test solutions in seawater, 150 ppm, 175 ppm, 200 ppm, 225 ppm, 250 ppm, 275 ppm, 300 ppm and 325 ppm. Due to the effect that temperature, pH and salinity have on the dissociation of ammonia into NH_4^+ and NH_3 (Whitfield 1974), the percentage of un-ionized ammonia (NH_3) in the test vessels was calculated for each stress test conducted. Temperature, pH and salinity were recorded using a Multiline P3 pH/Oxi-Set at the beginning and at the end of the test.

Un-ionized ammonia (NH_3) was estimated according to Fivelstad's (1988) equation:

$$\%NH3 = {}^{100}\!/\!_{1 + Anti\log[(9.245 + (0.002 * S)) + (0.034 * (24.85 - T)) - pH]}$$

where: S is salinity (‰), T is the temperature in degrees Celsius, pH is the average value of the pH readings taken at the start and end of the test.

Formalin stress test

Prior to conducting the formalin tests, a 1 000 ppm stock solution of formalin was made up in fresh seawater, which was then used to make up the desired concentrations. Five formalin concentrations, 12.5 ppm, 25 ppm, 37.5 ppm, 50 ppm and 62.5 ppm and a control of 0 ppm were tested.

For the starvation stress tests the larvae were placed into fresh seawater and the percent daily mortality was recorded over 144 hours. During the starvation tests water was exchanged once every 24 hours. Prior to the water exchange the new water was first equilibrated to the same temperature. This process was necessary to ensure that temperature shock did not act as a secondary stressor.

All LD_{50} values obtained in the respective stress tests were then correlated to the recorded egg and broodstock parameters.

Data analysis

All data sets were tested for normality using the Kolmogorov-Smirnov test at an error level of $p \leq 0.05$, prior to statistical analyses. If data were non-normally distributed or remained non-normally distributed after log or square-root transformations then non-parametric tests were used. Due to the small sample sizes non-parametric correlation tests (Spearman Rank Correlation) were used to establish if any relationships existed between parameters.

To determine whether egg colour, which consisted of ordinal data, affected hatch success or was itself dependent on the various broodstock parameters recorded, a non-parametric Kruskall-Wallis ANOVA by ranks was used. If a significant ($p \leq 0.05$) difference between groups was found, a Bonferroni correction was applied and the data were then tested pair-wise using the Man-Whitney U-test at an error level of $p \leq \left(\frac{0.05}{n}\right)$, where n = the number of pre-determined pair-wise comparisons.

Spearman rank correlations were used to quantify and describe the relationships between the egg and zoea lipid profiles as well to compare those to parameters such as hatch success, size of broodstock and the time taken to spawn. The null hypothesis was rejected at $p > 0.05$ in all tests. If significant correlations were found between parameters using the Spearman rank correlations, a regression analysis was undertaken to graphically present the relationships.

To establish whether the abundance of certain fatty acid classes differed significantly between egg and zoea samples, the non-parametric Sign test was used. This test was used instead of the Student's T Test, as sample sizes were small ($n < 30$). In all tests the null hypothesis was rejected at $p \leq 0.05$. Effect size measures tests (Cohen 1988) were also conducted on the fatty acid profiles of eggs and zoea to assess what percentage the proportions of individual fatty acids changed during the period of embryonic development.

All mortality data from the four stress tests were analysed using probit analysis. This allowed for the calculation of the LD_{50} value and upper and lower 95% confidence intervals for each batch of zoea subjected to the stress test. The calculated probits were then compared to the broodstock, egg and fatty acid parameters using Spearman rank correlations and the relationships plotted using regression analysis.

Results

Relationship between egg colour and egg quality

Egg colour ranged from pale yellow to orange-red. Of the 28 batches of eggs for which data for comparisons was recorded 14 % were pale yellow, 18 % were yellow, 43 % were orange-yellow, 18 % were orange and 7 % were orange-red in colour (Figure 4.2). Egg colour was not determined by the size of the female ($H_{4, 28} = 5.00$; $1 \geq p \geq 0.28$) as shown in Figure 4.3, nor was it related to the time females spent in captivity prior to spawning ($H_{4, 26} = 7.48$; $1 \geq p \geq 0.11$) as shown in Figure 4.4, and had no effect on the hatch success rate of the eggs ($H_{4, 28} = 6.46$; $1 \geq p \geq 0.16$). The hatch success rate of the 28 batches of eggs recorded ranged from 68 – 97 %. The mean % hatch rate of the different coloured egg batches is shown in Figure 4.5

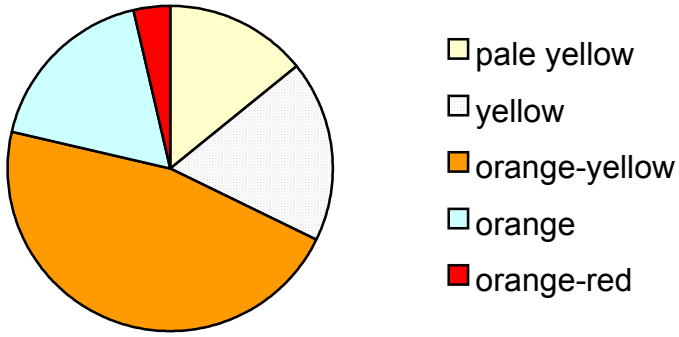


Figure 4.2: Proportion of berried crabs with different colour eggs. (n =28)

Hatch synchronicity varied greatly between batches. The time taken to complete the hatching process ranged from 45mins to 18 hours. In total 22 % of the crabs completed the hatching process in less than 1 hour, 36 % between 1-2 hours, 11 % between 2-3 hours, while the remainder (31 %) completed the hatching process between 3-18 hours.

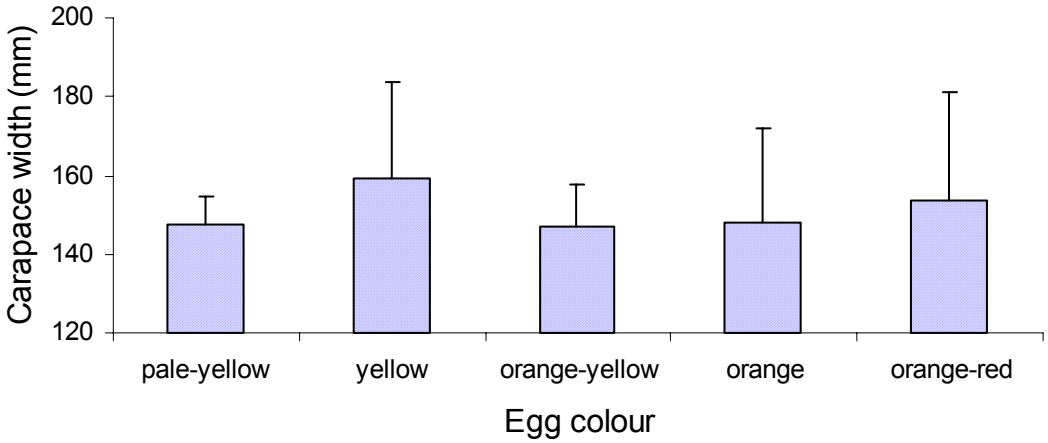


Figure 4.3: Relationship between female size (mean and S.D.) and the different coloured egg batches (n = 28)

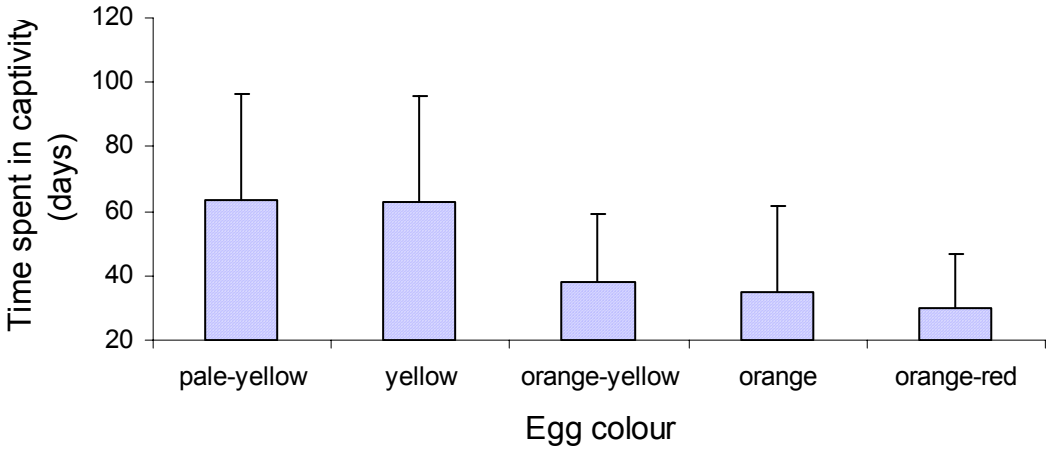


Figure 4.4: Relationship between the time females spent in captivity prior to spawning (mean and S.D.) and different coloured egg batches (n = 28)

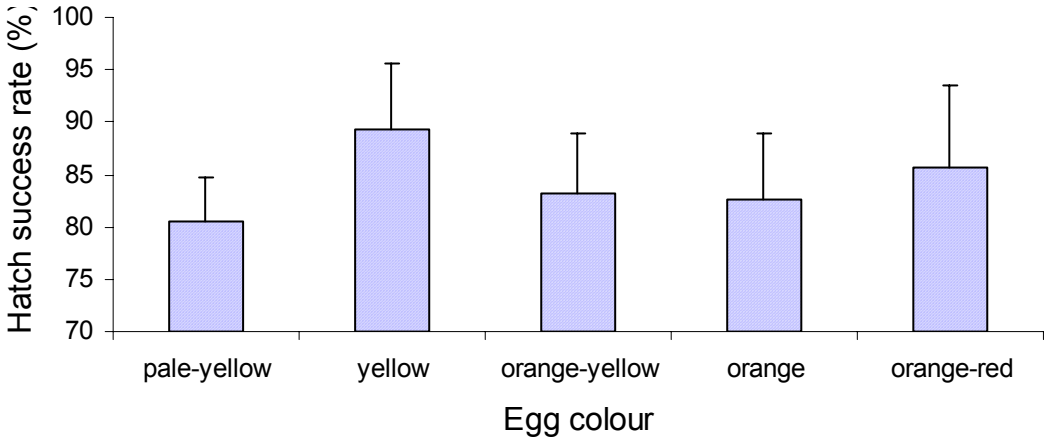


Figure 4.5: Relationship between hatch success rate (mean and S.D.) and the different coloured egg batches (n = 28)

There were no significant relationships ($0.05 \leq p \leq 1$) between hatch synchronicity and any of the recorded broodstock and egg parameters (Table 4.1). Hatch synchronicity was also not affected by egg colour ($H_{4, 28} = 7.84$; $1 \geq p \geq 0.1$) (Figure 4.6).

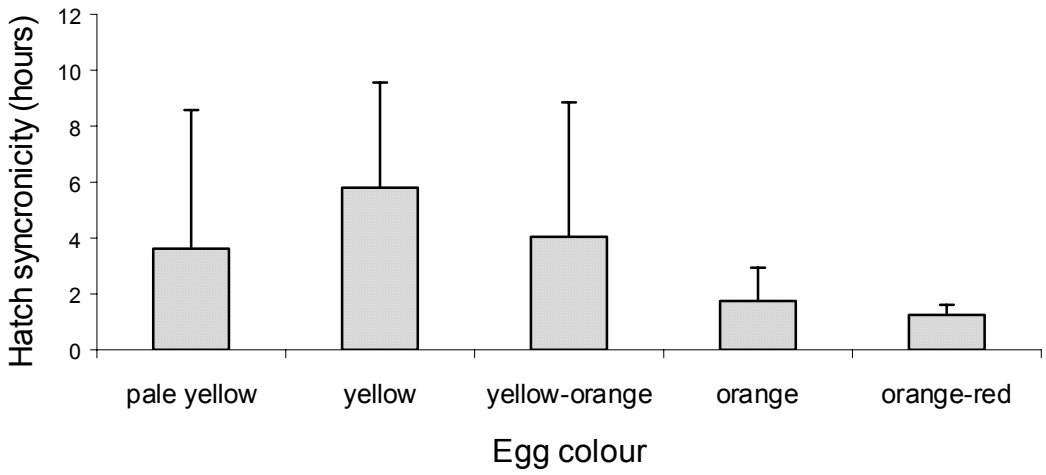


Figure 4.6: Relationship between hatch synchronicity and egg colour (n = 28)

Table 4.1: Relationship between hatch synchronicity and various crab and egg parameters (n = 28)

Hatch Synchronicity Vs.	**P-Value**
Carapace width (mm)	0.792
Time (days) in captivity	0.832
Egg colour	0.187
Hatch Success (%)	0.587
Fecundity	0.487

The results therefore suggest that egg colour cannot be used as a simple indicator of egg quality.

The relationship between the fatty acid profiles of egg and zoea and egg quality

Given that the females in captivity were fed on a standardized feeding regime (see Chapter 2) the fatty acid profiles of the different feeds are presented purely as a reference for future comparative studies. There were significant differences in the fatty acid profiles of the various broodstock feed components. Docosahexaenoic acid

(DHA) ranged from 42.3 % of the total fatty acid content in squid to 3.4% for snails (Table 4.2). The (n-6):(n-3) ratio also varied between the diets with squid having a ratio of 0.03:1 while the prawn and snail diets had (n-6):(n-3) ratios of 1.48:1 and 2.81:1, respectively. In all diets the 16:0 (Palmitic acid); 18:1n-9 (Oleic acid); 20:5n-3 (Eicosappentaenoic acid) and 22:6n-3 (Docosahexaenoic acid) fatty acids were the most prevalent. In the snail diet 20:4n-6 (Arachidonic acid) was the predominant fatty acid with over six times the amount in the other feeds (Table 4.2).

Table 4.2: Fatty acid profiles of the feed ingredients (Values are expressed as % Area)

Diet composition	**Fish roe**	**Squid**	**Prawn**	**Mangrove snails**
Total lipid (% dry weight)	25.07	8.63	15.66	2.79
Protein (% dry weight)	68.43	78.41±0.2	77.29	60.42
Major fatty acids (Area%)				
Saturated				
14:0	1.6	1.6	0.9	2.4
16:0	17.1	25.4	21.2	13.8
18:0	2.0	3.5	5.7	8.8
Monounsaturated				
16:1 *n*-7	4.0	0.8	2.4	1.4
18:1 *n*-9	21.7	1.8	21.9	4.8
18:1 *n*-7	3.5	1.2	3.4	2.8
20:1 *n*-9	1.9	2.8	4.0	*
Polyunsaturated				
18:2 *n*-6	1.5	0.7	18.5	5.0
20:4 *n*-6	2.5	1.0	1.0	15.9
20:4 *n*-3	0.7	0.1	0.2	0.0
20:5 *n*-3 (EPA)	7.8	12.4	4.8	5.3
22:5 *n*-3	1.5	0.5	0.9	1.6
22:6 *n*-3 (DHA)	25.6	42.3	7.2	3.4
Σ omega-3 fatty acids	35.7	56.3	13.4	10.5
Σ omega-6 fatty acids	5.1	1.9	19.9	29.0
DHA:EPA ratio	3.27:1	3.38:1	1.5:1	0.6:1
(*n*-6):(*n*-3) ratio	0.14:1	0.03:1	1.48:1	2.81:1

The fatty acid (FA) composition of eggs and zoea are presented in Table 4.3. In all samples the predominant fatty acids consisted of 16:0, 18:0, 18:1n-9, 20:5n-3 and 22:6n-3. The percentage of 20:1n-9 was similar in the egg and zoea samples. Significant differences ($p \leq 0.05$) for 16:1n-7 and 18:1n-7 were, however, found

between the egg and zoea samples. Most polyunsaturated fatty acid (PUFA) levels in the egg and zoea samples showed significant differences ($p \leq 0.05$), with the levels of 20:4n-3, 20:4n-6 and EPA increasing from eggs to zoea, while the 22:5n-3 and DHA levels decreased (Table 4.3). The percent change in the proportions of individual fatty acids between eggs and zoea, using Cohen's effect size measures tests, are presented in Table 4.4.

Table 4.3: Fatty acid profiles of eggs (n = 28) and zoea (n = 16) (Values are expressed as mg/g DW and presented as mean ± standard deviation)

Fatty acids	Eggs	Zoea
Saturated		
14:0	2.2±0.5 [a]	1.1±0.6 [b]
16:0	20.9±1.5 [a]	20.1±3.2 [a]
18:0	8.2±1.1 [a]	10.9±2.2 [b]
Monounsaturated		
16:1 *n*-7	6.5±1.3 [a]	2.8±0.6 [b]
18:1 *n*-9	11.3±1.2 [a]	11.1±2.5 [a]
18:1 *n*-7	3.9±0.6 [a]	4.8±1.0 [b]
20:1 *n*-9	1.8±0.5 [a]	1.8±0.8 [a]
Polyunsaturated		
18:2 *n*-6	2.1±0.8 [a]	2.1±1.3 [a]
20:4 *n*-6	2.2±0.8 [a]	3.4±1.5 [b]
20:4 *n*-3	0.2±0.1 [a]	0.7±0.2 [b]
20:5 *n*-3 (EPA)	9.2±1.9 [a]	13.0±2.7 [b]
22:5 *n*-3	1.2±0.6 [a]	0.7±0.4 [a]
22:6 *n*-3 (DHA)	16.8±4.1 [a]	13.0±4.7 [a]
Σ omega-3 fatty acids	27.6±5.0 [a]	28.2±5.6 [a]
Σ omega-6 fatty acids	5.3±1.4 [a]	6.1±1.9 [a]
DHA:EPA ratio	1.9±0.5:1 [a]	1.0±0.4: [b]
(*n*-6):(*n*-3) ratio	0.2±0.09:1 [a]	0.2±0.1:1 [a]

Within a row, values with different superscript letters are significantly different ($p \leq 0.05$).

EPA levels showed a significant difference ($p \leq 0.05$) between the egg and zoea samples. EPA levels increased from 9.2 % of the total FA in eggs to 13 % in zoea. The DHA:EPA ratios also differed significantly ($p \leq 0.05$) between eggs and zoea, with the zoea DHA:EPA ratio being half that of the eggs. However, there was no significant difference in the (n - 6):(n - 3) ratios in the eggs and zoea. There was a significant difference in total mg FAME/g DW between the eggs and zoea. The eggs

contained up to four times greater amounts of fatty acids (163 ± 13.4 mg/g DW) than the zoea (48 ± 11.6 mg/g DW).

Table 4.4: Changes in the proportions of individual fatty acids between eggs and newly hatched zoea using Cohen's effect size measures test (n = 16)

Fatty acids	**% Decrease (range) in the proportions of individual fatty acids from eggs to zoea**	**% Increase (range) in the proportions of individual fatty acids from eggs to zoea**
Saturated		
14:0	77-79	
16:0	7-14	
18:0		70-73
Monounsaturated		
16:1 *n*-7	>81	
18:1 *n*-9	0-7	
18:1 *n*-7		51-55
20:1 *n*-9		0-7
Polyunsaturated		
18:2 *n*-6	7-14	
20:4 *n*-6		51-55
20:4 *n*-3		14-21
20:5 *n*-3 (EPA)		68-70
22:5 *n*-3	38-43	
22:6 *n*-3 (DHA)	33-38	
Σ omega-3 fatty acids		14-21
Σ omega-6 fatty acids		27-33

The statistical analyses showed that the fatty acid profiles of eggs were related to some of the egg and broodstock parameters as follows. EPA and the Σ omega-3 in the eggs were significantly correlated ($p = 0.0028$ and $p = 0.011$) to female size. Both decreased with an increase in crab size (Fig 4.7). The relationships are best described by the linear equations: $EPA = 1.91 - 0.01CW$ ($r^2 = 0.35$; $p = 0.0028$) and $\Sigma\ omega\text{-}3 = 2.41 - 0.005CW$ ($r^2 = 0.26$; $p = 0.011$), where CW = carapace width (mm).

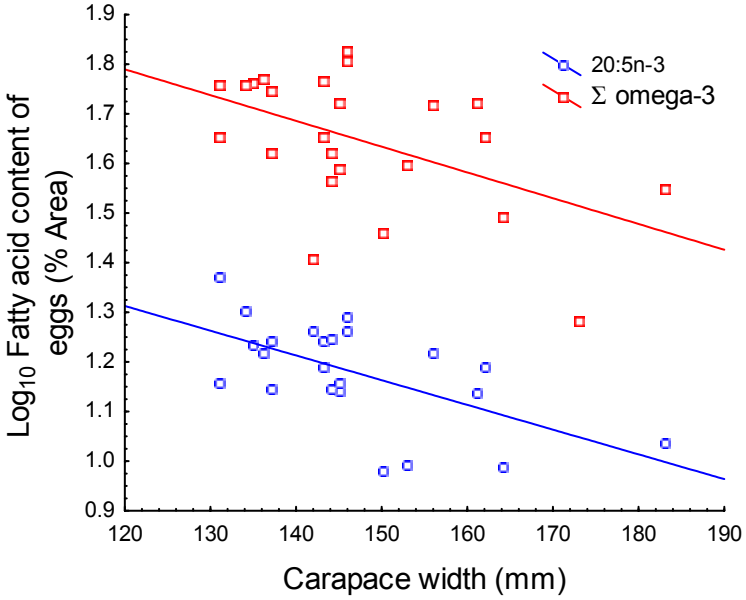


Figure 4.7: Relationship between female size and EPA and the Σ omega-3 fatty acids in the eggs. (n = 28)

Hatch success, individual egg mass and the time females spent in captivity prior to spawning showed no correlation ($0.05 \leq p \leq 1$) to the fatty acid content in the eggs. The fatty acid profiles also showed no correlation ($0.05 \leq p \leq 1$) to egg colour (Figure 4.8, Table 4.5).

Table 4.5: The relationship between essential fatty acids and the five different coloured egg batches using Kruskal-Wallis ANOVA of ranks. Significant relationships are indicated at $p \leq 0.05$

	Kruskal-Wallis	**p-value**
ARA	$H_{4, 24} = 8.12$	0.09
EPA	$H_{4, 23} = 8.21$	0.08
DHA	$H_{4, 24} = 3.53$	0.47
DHA:EPA ratio	$H_{4, 23} = 4.07$	0.40
Σ omega-3 fatty acids	$H_{4, 24} = 2.51$	0.64
Σ omega-6 fatty acids	$H_{4, 24} = 4.76$	0.31

Egg size at extrusion showed no correlation ($1 \geq p \leq 0.05$) to any of the essential fatty acids found in the eggs. The size of the egg just prior to hatching was however significantly correlated to a number of fatty acids in both the egg and zoea samples. The essential fatty acid EPA was significantly correlated ($p = 0.015$) to final egg size with larger eggs having a higher EPA content (Figure 4.9). This relationship is best

represented by the linear equation: $EPA = -92.69 + 272.17 ESH$, where ESH = egg size at hatch.

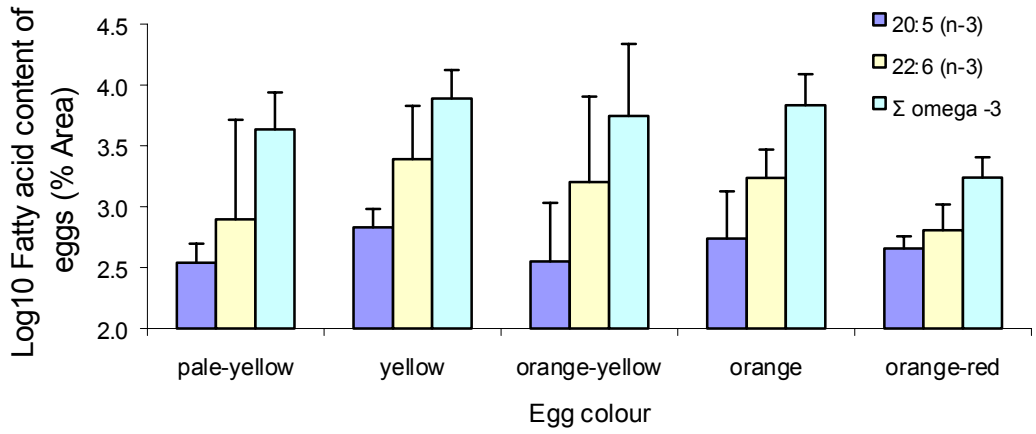


Figure 4.8: Relationship between the essential fatty acids 20:5 (n-3), 22:6 (n-3) and the Σ omega – 3 fatty acids in different coloured egg batches (n = 28)

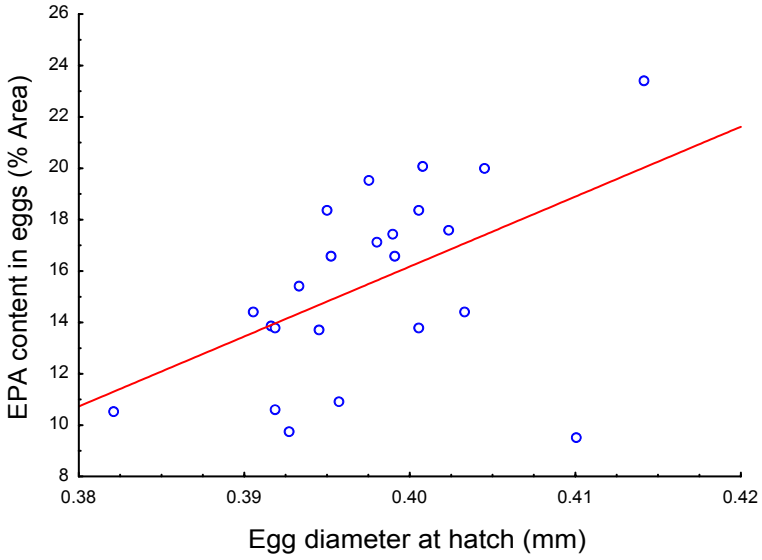


Figure 4.9: EPA content in newly extruded eggs and its relationship to egg size at hatch ($r^2 = 0.249$, p = 0.015, n = 26)

During embryonic development the fatty acid profiles changed significantly ($p \leq 0.05$) within the eggs. The proportions of EPA, DHA, ARA and the DHA:EPA ratio all changed significantly ($p \leq 0.05$) during embryonic development. The proportion of EPA and ARA increased in the egg during embryonic development (Figure 4.10) while DHA and the DHA:EPA ratio decreased (Figure 4.11). There was however, no significant change in the Σ omega-3 and Σ omega-6 fatty acids.

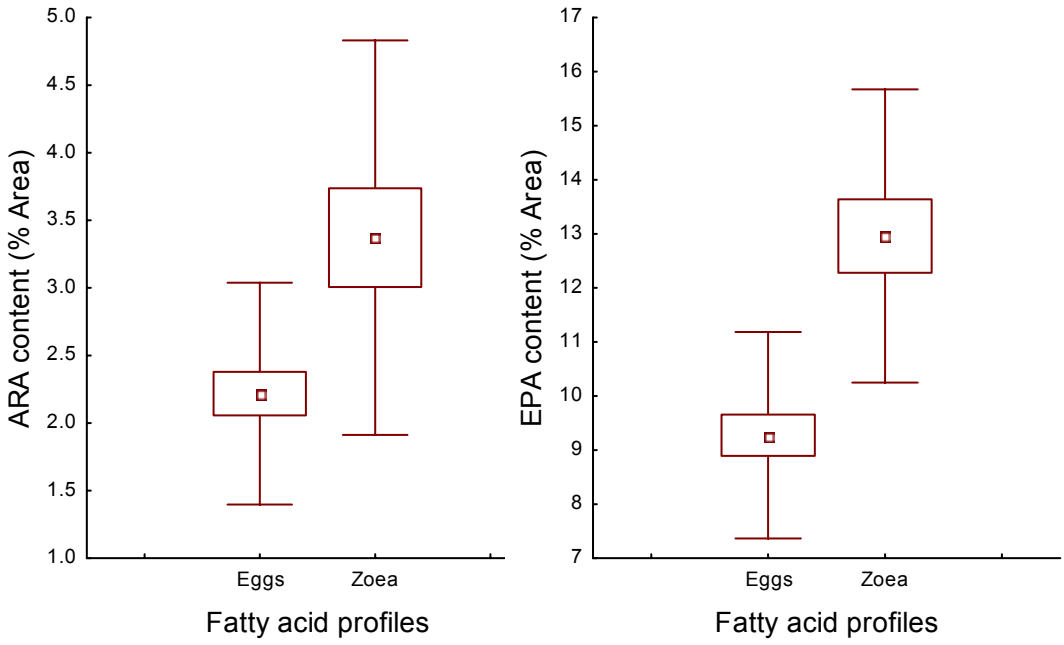


Figure 4.10: Changes (mean ± S.D.) in the proportions of ARA and EPA in newly extruded eggs and newly hatched zoea (ARA, p = 0.00; EPA, p = 0.00, n = 28 for eggs and n = 16 for zoea)

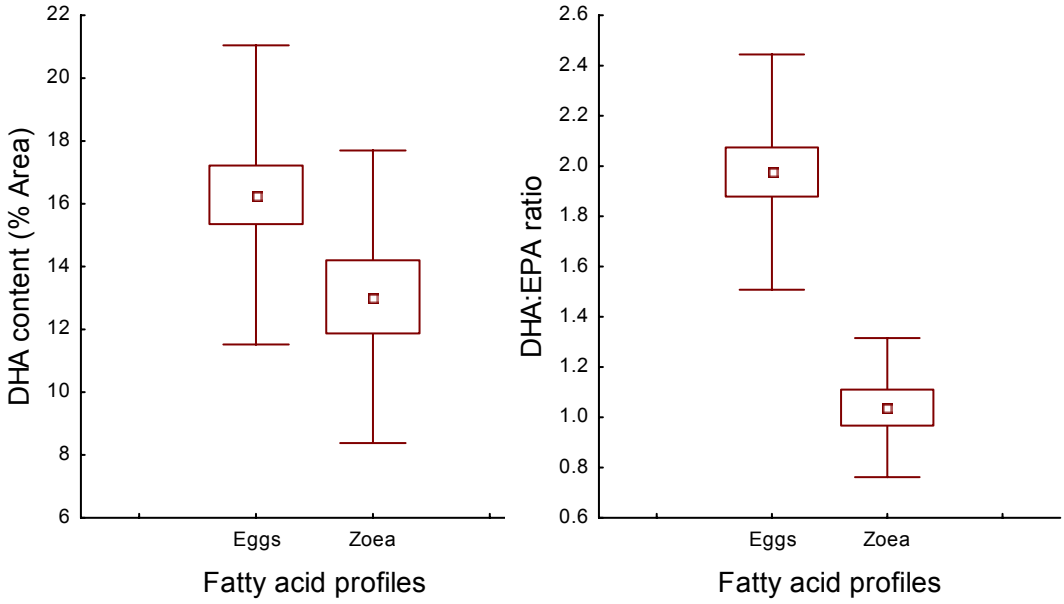


Figure 4.11: Changes (mean ± S.D.) in the proportions of DHA and the DHA:EPA ratio in newly extruded eggs and newly hatched zoea (DHA, p = 0.04; DHA:EPA ratio, p = 0.00, n = 28 for eggs and n = 16 for zoea)

There was no correlation between the essential fatty acid profile of newly hatched zoea ($0.05 \leq p \leq 1$) and any of the recorded broodstock and egg parameters i.e. crab

size, length of time in captivity, egg size at hatch and hatch success. However, there was a significant relationship ($p = 0.03$) between egg size at extrusion and EPA content in newly hatched zoea, but there was no relationship ($p \geq 0.05$) between egg size at extrusion and the other essential fatty acids (Table 4.6).

Table 4.6: Relationship between essential fatty acids in newly hatched zoea and the recorded broodstock and egg parameters. Significant relationships are indicated at $p \leq 0.05$ (n = 16)

	p-Values					
	18:3(n-3)	**20:4(n-6)**	**EPA**	**DHA**	**(n-6)/(n-3)**	**DHA/EPA**
Crab size	0.56	0.92	0.24	0.24	0.87	0.71
Incubation period	0.74	0.77	0.68	0.27	0.10	0.27
Egg size at extrusion	0.66	0.62	0.03*	0.46	0.95	0.59
Egg size at hatch	0.54	0.46	0.64	0.81	0.39	0.98
Hatch success	0.65	0.72	0.43	0.33	0.23	0.57

* Indicates those fatty acids which are significantly related to the recorded parameters.

There was no significant relationship between the essential fatty acid profiles of newly hatched zoea that originated from different coloured egg batches (Table 4.7; Figure 4.12).

Table 4.7: Relationships between the essential fatty acids of newly hatched zoea and egg colour using Kruskal-Wallis ANOVA of ranks. Significant relationships are indicated at $p \leq 0.05$ (n = 16)

	Kruskal-Wallis	**p-value**
ARA	$H_{2,16} = 0.34$	0.825
EPA	$H_{2,16} = 5.71$	0.058
DHA	$H_{2,16} = 0.26$	0.877
DHA:EPA ratio	$H_{2,16} = 3.41$	0.181
Σ omega-3 fatty acids	$H_{2,16} = 2.06$	0.356
Σ omega-6 fatty acids	$H_{2,16} = 0.52$	0.771

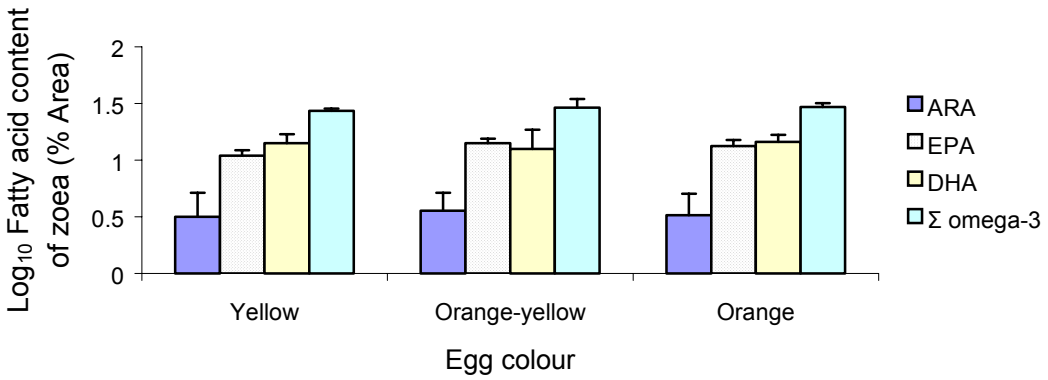


Figure 4.12: Relationship between the essential fatty acids ARA, EPA, DHA and Σ omega-3 fatty acids in zoea originating from different coloured egg batches (n = 16)

Stress test results

Starvation test

Using probit analysis an average LD_{50} value of 64 hours was recorded for the 28 starvation tests with upper and lower 95% confidence limits of 71.96 and 52.57 hours, respectively (Figure 4.13).

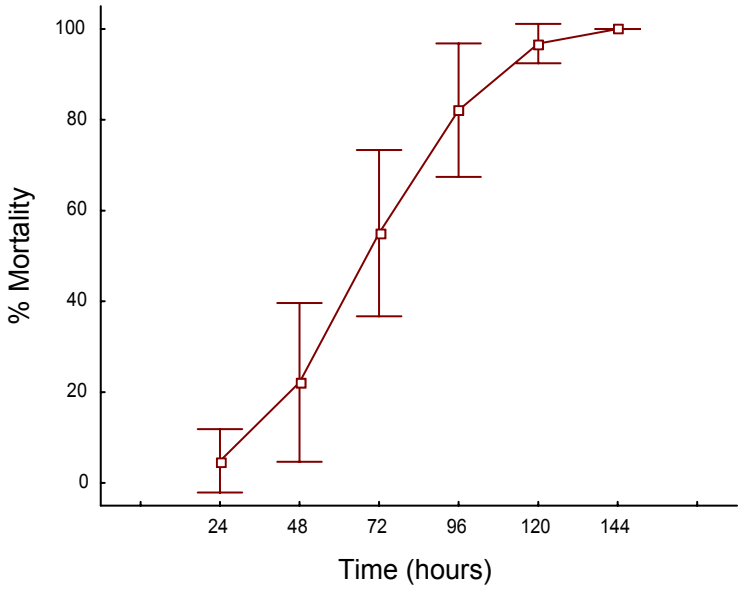


Figure 4.13: Average daily mortality curve for *Scylla serrata* zoea during the starvation tests. Each data point is the mean with 95% confidence intervals (n = 28)

The behaviour of the zoea for the first 48 hrs was "normal", where after swimming activity became sporadic. After 72-hours swimming was rarely observed and was restricted to short bursts. In all tests no zoea survived for longer than 144 hours.

There were no significant relationships ($0.05 \leq p \leq 1$) between the starvation LD_{50} values and the recorded broodstock and egg parameters. There was also no significant relationship between the starvation LD_{50} values and the fatty acid composition of the eggs. Similarly no relationship could be found between the starvation LD_{50} values and zoea originating from different coloured egg batches ($H_{2,16} = 0.98$; $1 \geq p \geq 0.61$). However, there were significant correlations between the DHA ($p = 0.02$) and Σ omega-3 fatty acids ($p = 0.043$) in newly hatched zoea and survival in the starvation tests (Figure 4.14). Higher levels of DHA and Σ omega-3 fatty acids in the newly hatched zoea resulted in stronger larvae.

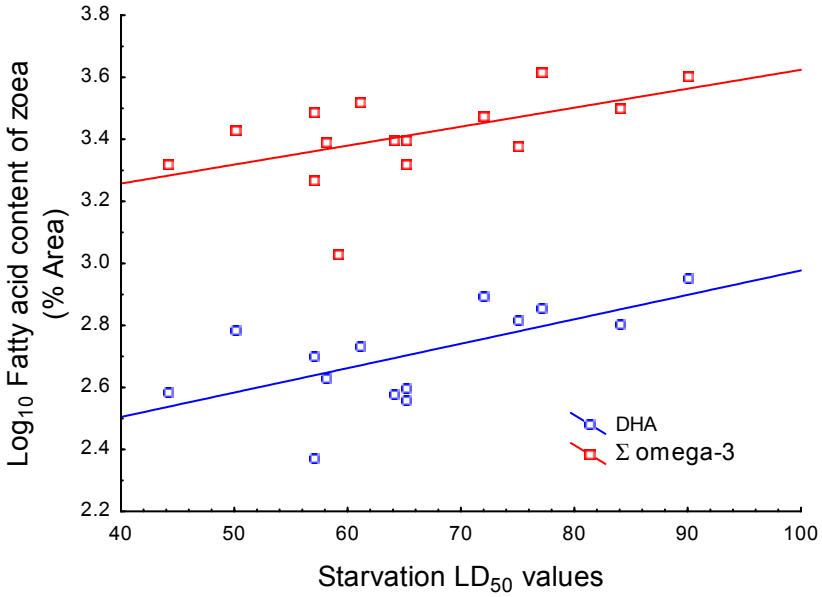


Figure 4.14: Relationship between starvation LD_{50} values and DHA and the Σ omega-3 fatty acids in newly hatched zoea (n = 16)

Salinity

Using probit analysis the average salinity LD_{50} was 42 ‰ with upper and lower 95 % confidence intervals of 39.5 ‰ and 44.5 ‰, respectively (Figure 4.15). Throughout the duration of the salinity stress tests the zoea in the control treatments at 32-34 ‰ remained unaffected and exhibited active swimming behaviour. At 40 ‰ the zoea remained active for up to 12 hours. At 45 ‰ the zoea ceased swimming after 3 hours, at 50 ‰ after 90 minutes and at 55 ‰ after 30 minutes.

Of all the recorded broodstock and egg parameters only crab size showed a significant correlation ($p = 0.02$) with the salinity LD_{50} values. Zoea originating from larger crabs had lower LD_{50} values than those originating from smaller females. This relationship is most likely spurious as there was no correlation between crab size and egg size. Essential fatty acid profiles of eggs, egg colour and newly hatched zoea showed no correlation ($0.05 \leq p \leq 1$) with the salinity LD_{50} values.

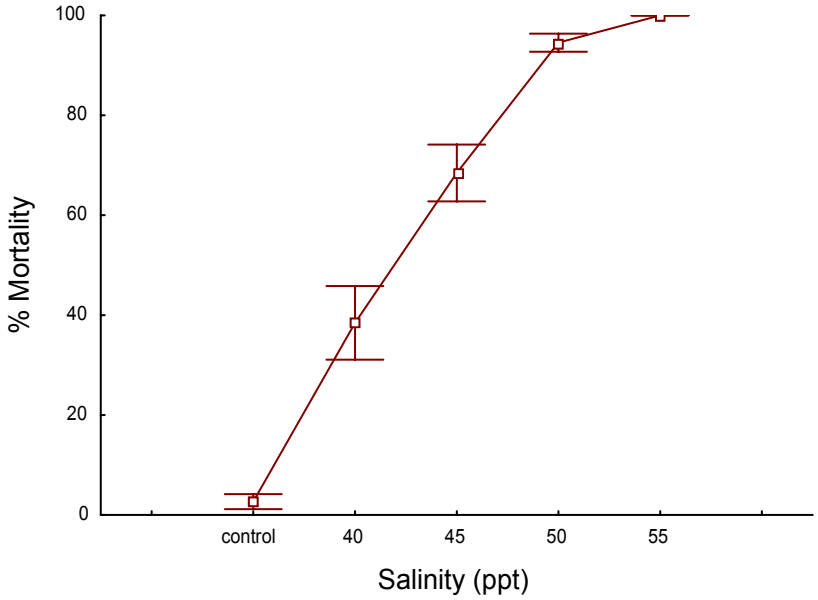


Figure 4.15: Dose effect curve for *Scylla serrata* zoea exposed to different salinities after 24 hours. Each data point is the mean with 95% confidence intervals (n =28)

Ammonia

During the ammonia stress tests NH_3 levels ranged between 0 mg in the control to 79.8 mg/l (total ammonia). The average LD_{50} value was 40 mg/l NH_3 with lower and upper 95 % confidence intervals of 38.86 mg/l and 42.16 mg/l, respectively (Figure 4.16). Temperature did not fluctuate by more than $1^{o}C$ during any of the tests (within 24-hours).

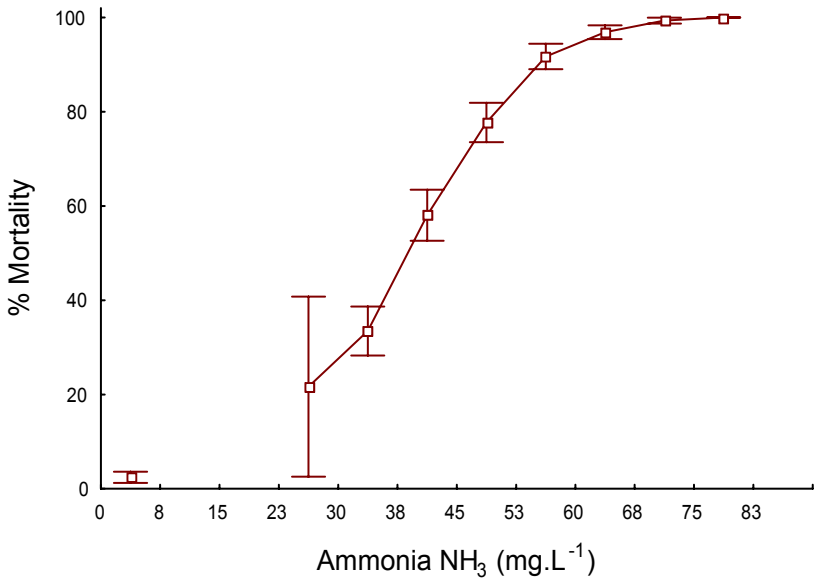


Figure 4.16: Dose effect curve for *Scylla serrata* zoea exposed to ammonia after 24 hours. Each data point is the mean with 95% confidence intervals (n =28)

During the ammonia tests zoea activity remained normal in the control and in the 20 and 30 mg/l NH_3 test solutions for the duration of the tests. At the higher test concentrations swimming activity of the zoea ceased within 3-hours of being introduced into the test vessels, whereafter the activity consisted of short bursts of swimming before settling motionless onto the bottom of the test vessels. The ammonia LD_{50} values showed no correlation ($0.05 \leq p \leq 1$) with the recorded broodstock and egg parameters. Similarly no significant correlations were found between the egg and zoea fatty acid profiles or egg colour and the ammonia LD_{50} values.

Formalin

The average LD_{50} value for the formalin tests was 40.5 ppm, with lower and upper 95% confidence intervals of 30 ppm and 51 ppm, respectively (Figure 4.17). In most of the tests the zoea in the 12.5 ppm solution remained active for the duration of the 24-hour tests. In the 25 ppm treatments swimming activity stopped after approximately 9 hours, while at the higher concentrations swimming activity stopped within 1 hour.

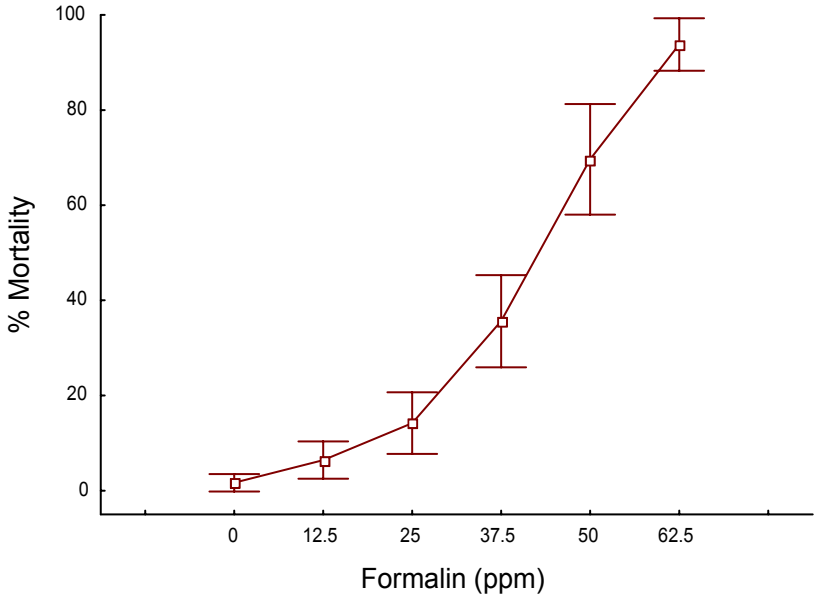


Figure 4.17: Dose effect curve for *Scylla serrata* zoea exposed to formalin after 24 hours. Each data point is the mean with 95% confidence intervals (n =28)

The formalin LD_{50} values showed significant relationships with some of the recorded broodstock and egg parameters. Female size and time spent in captivity prior to spawning were significantly ($p \leq 0.05$) correlated to the formalin LD_{50} values. The formalin LD_{50} values decreased with an increase in CW ($r^2 = 0.4$; $p = 0.001$) and the time females spent in captivity prior to spawning ($r^2 = 0.28$; $p = 0.008$) (Figure 4.18). There was no relationship between larval survival in the formalin tests and the different coloured egg batches ($1 \geq p \geq 0.05$). The results suggest that smaller females produce better quality eggs and that egg quality decreases with time spent in captivity.

Significant correlations were found between the formalin LD_{50} values and the EPA and the Σ omega-3 content of the eggs. In both, an increase in the levels of EPA and Σ omega-3 in the eggs was reflected by an increase in the formalin LD_{50} values (Figure 4.19). These relationships were best represented by the linear equations: $EPA = 2.33 + 0.01 ForLD_{50}$ where $ForLD_{50}$ = the formalin LD_{50} value ($r^2 = 0.33$; $p = 0.003$), and $\Sigma\ omega\text{-}3 = 3.29 + 0.01 ForLD_{50}$ ($r^2 = 0.27$; $p = 0.007$). The relationship between the Σ omega-3 content of zoea and the formalin LD_{50} values was also significant ($p = 0.018$). An increase in the level of Σ omega-3 in the zoea was reflected by higher formalin LD_{50} values. The results suggest that higher EPA and Σ

omega-3 fatty acid levels in the eggs and higher Σ omega-3 fatty acids in stage 1 zoea, result in stronger larvae.

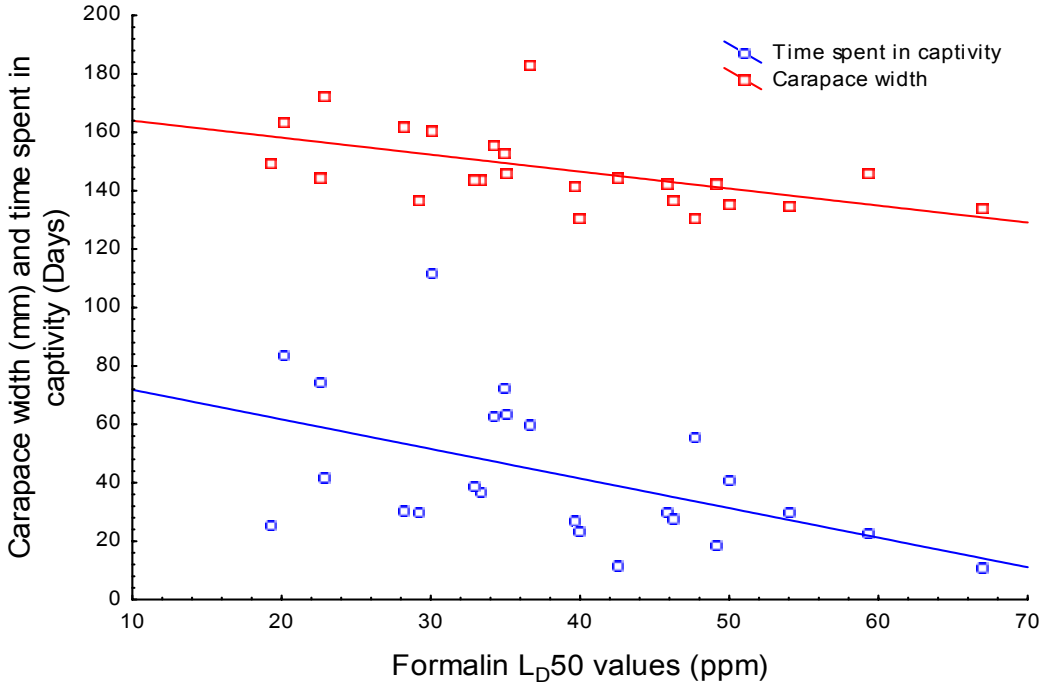


Figure 4.18: Relationship between formalin LD_{50} values and female size and time spent in captivity (n = 28)

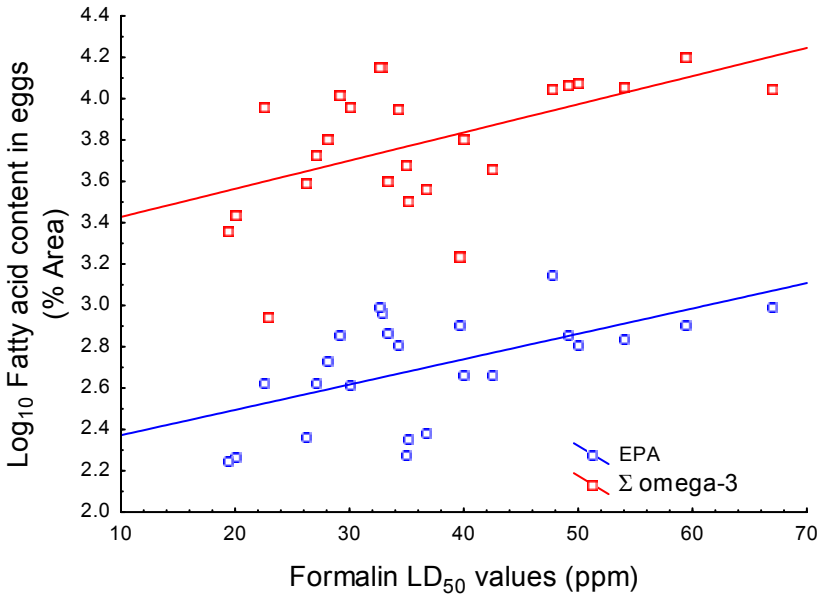


Figure 4.19: Relationship between 24-hour formalin LD_{50} values and EPA and Σ omega-3 content in newly extruded eggs (n = 28)

Discussion

The colour of the crab eggs ranged from pale yellow to orange-red. Previously egg colour was considered to be an indicator of egg quality in *Scylla serrata* (Ong 1966, DuPlessis 1971). In this study there were no statistical differences between hatch rates of different coloured eggs. Moreover, embryonic development proceeded normally and showed no differences between different colour egg batches (see Chapter 3). This is similar to what has been found for the crayfish, *Astacus leptodacylus* (Berticat *et al.* 2000) in which egg colour varies from orange to brown, and for which embryonic development was identical irrespective of egg colour as was the carotenoid content. In the present study carotenoid content of the eggs was not measured.

Carotenoids, in particular astaxanthin, are responsible for the orange-red colour in eggs and larvae, and has been shown to affect stress resistance in *Penaeus monodon* postlarvae (Merchie *et al.* 1998). These carotenoids act as antioxidants by inactivating damaged free radicals produced by normal cellular activity and from various stressors (Chew 1995). In fish, such as the red sea bream, *Pagrus major*, diets containing higher levels of astaxanthins fed to broodstock just prior to spawning led to improved larval vitality and hatch rates (Watanabe *et al.* 1991). The general conclusion from this is that carotenoid content is in fact an indicator of egg quality and by implication orange-red coloured eggs should be the best. In this study, however, it was shown that egg colour was not related to crab size and time spent in captivity and that there were no statistical differences in hatch rates or hatch synchronicity between different colour egg batches. Similarly the results of the stress tests showed no significant difference between zoeal survival from different coloured egg batches. These observations suggest that the hypothesis of egg colour being an indicator of egg quality be rejected.

It has been suggested that the hatching process in brachyuran crabs is initiated by a chemical/pheromone stimulus released by the female (Forward *et al.* 1982, Forward and Lohmann 1983). This stimulus results in the flexing of the abdomen by the female (Rittschof *et al.* 1989), which helps to synchronise hatching by mechanically stimulating and rupturing the egg membrane (Davis 1981). For most brachyuran crabs

the hatching process is a brief and seemingly synchronised event lasting only a few minutes until all the eggs in the bolus have hatched (Tankersley *et al.* 2002).

In this study the hatching process was also mediated by the flexing of the abdomen but lasted between 1 and 18 hours. The duration of the hatching process was independent of egg colour, female size, fecundity or the time that the females spent in captivity prior to spawning. Moreover, there was no correlation between hatch success rate and the duration of the hatching process. The stress tests also indicated that that there was no difference in zoeal quality among batches in which hatching was highly or poorly synchronised.

These findings contradict the suggestion made by David Mann (Bribie Island Aquaculture Research Centre, Queensland, Australia. pers. comm.), that delayed hatching (poor hatch synchronicity) is an indicator of inferior egg quality, manifested by low hatch success and high larval mortalities. Newly hatched *S. serrata* zoea have no endogenous food reserves and need an adequate supply of high quality food organisms immediately after hatching (Fielder 1995). The high larval mortality in batches of eggs with poor hatch synchronicity may therefore simply be a manifestation of starvation stress in the early hatching larvae and not an indicator of poor egg quality. Mann and Parlato (1995) found that starvation of S. *serrata* zoea during the first 24 hours post hatch did not affect survival or development to the second zoea stage, but emphasised that more work is required to define critical periods in larval feeding and to determine the later consequences of early food deprivation for mud crab larvae. This study by Mann and Parlato (1995) does not, however, invalidate the suggestion made here that hatch synchronicity is not an indicator of egg quality.

It is generally accepted that broodstock feed quality and quantity are important determinants of egg quality and viability (De Culuwé *et al.* 1995_a). This has led to numerous investigations on the relationships between broodstock diet and the resulting lipid and fatty acid composition of eggs and larvae (De Caluwé *et al.* 1995_a, Furutia *et al.* 2000, Millamena and Quinitio 2000). Broodstock feeds were analysed for fatty acid content and this was related to the fatty acid content of the eggs and zoea. Although the effects of diet composition on the fatty acid composition and

content of the eggs and zoea was not in the scope of this study, the EFA content of the various feeds was higher than in the eggs and zoea. Further research is required to determine the optimal inclusion levels and proportions of the Σ omega-3 and Σ omega-6 fatty acids in *S. serrata* broodstock diets (Harrison 1990).

The major fatty acids found in the eggs and zoea of *S. serrata* in this study were the neutral lipids (non essential fatty acids) such as 16:0 and the omega-9 fatty acids, and the essential fatty acids 20:5 n-3 (EPA), 22:6 n-6 (DHA) and 20:4 n-6 (ARA). The levels of individual fatty acids did, however, vary between batches of eggs, e.g. DHA varied by over 4% around the mean between batches. High variability in fatty acid composition has also been found between different batches of turbot (*Scophthalmus maximus L.*) eggs (Peleteiro *et al.* 1995). As in this study Peleteiro *et al.* (1995) could also not draw any correlation between the fatty acid composition of the eggs and egg quality.

In all batches of eggs the Σ omega-3 and Σ omega-6 fatty acids, in relation to the total fatty acid content, remained relatively constant between newly extruded eggs and newly hatched zoea. This was, however, not the case with DHA, EPA and the DHA:EPA ratio between egg and zoea samples. During embryonic development the proportion of DHA in the total fatty acid profile decreased from egg to zoea, while the proportion of EPA increased. Feeding crustacean larvae with DHA and EPA enriched diets leads to improved survival and growth rates (Levine and Sulkin 1984, Kontara *et al.* 1995). This provides evidence that DHA and EPA in particular are essential for zoeal development. The question that arises is, if DHA and EPA are the two most important essential fatty acids, then what are the required levels upon which to assess egg batches as being of poor or good quality?

Saturated fatty acids, such as 14:0 (Myristic) and 16:0 (Palmitic) and monounsaturated fatty acids, such as 16:1n-7 are abundant in detritus and natural food organisms which are consumed by marine crustaceans such as crabs and shrimp (Jeckel *et al.* 1989). In addition, these fatty acids are synthesised during ovarian maturation (Clarke 1982) and incorporated into the egg yolk making then available for embryonic development. The omega-3 HUFA fatty acids in particular EPA and DHA either cannot be synthesised *de novo* (Clarke 1982) or may be synthesised to a

limited extent through the conversion of 18:2 (n-6) and 18:3 (n-3) to C_{20} and C_{22} HUFA's, respectively (Mourente 1996). The results presented here suggest that EPA appears to have been set aside for its role in later larval development, while other omega-3 fatty acids such as 22:5n-3 and DHA appear to be used during embryonic development along with the non-essential fatty acids such 14:0 and 16:1 (n-7). The proportion of 16:1n-7 and 14:0 dropped by over 81% and 77-79% respectively from levels found in the eggs to those found in zoea, while the proportion of EPA and ARA in the fatty acid profiles of zoea increased by between 68-70% and 51-55%, respectively, from the levels found in the eggs.

There was a significant negative correlation between crab size and the EPA content in eggs - EPA levels decreased with increasing crab size. The time that the female spent in captivity, however, had no effect on the EPA content of eggs. The necessity of adequate amounts of EPA for larval development in brachyuran crabs (Levine and Sulkin 1984) and other species is well documented (Lavens *et al.* 1991, Alava *et al.* 1993). The implication of this finding is to choose optimal sized crabs for broodstock purposes, which will produce eggs with high EPA content. From results found in this study the optimal size for broodstock females should range between 125 – 145 mm carapace width.

Egg size at hatch was significantly correlated to the EPA content of the eggs. The level of EPA increased with increasing egg size. The fatty acid profiles of newly hatched zoea did show a positive correlation between EPA content and size of egg at extrusion. There was, however, no correlation between the fatty acid profiles of newly hatched zoea and the other recorded egg and broodstock parameters. The implication of this finding is that EPA is in fact an important determinant of egg quality, and appears to be set aside during embryonic development for use by the newly hatched zoea. This finding highlights the need to ensure high levels of EPA in newly extruded eggs either by selecting optimal sized broodstock or through broodstock nutrition.

Stress tests have been extensively used to determine larval quality in crustaceans (Bauman & Jamandre 1990, Briggs 1992, Tayamen and Brown 1999) and to establish dietary and environmental effects on larval survival (Tackaert *et al.* 1989, Rees *et al.* 1994). In this study stress tests were used in a retrospective manner to determine

whether any of the broodstock and egg parameters could be used as a reliable indicator of egg quality. These parameters included the time broodstock spent in captivity, crab size, egg colour, egg size, fatty acid content of eggs, fatty acid content in zoea, hatch success rate and hatch synchronicity.

The starvation tests showed no significant correlations to any of the broodstock and egg parameters but was significantly correlated to the DHA and Σ omega-3 content of newly hatched zoea. The starvation test results closely resemble those found by Mann and Parlato (1995), who recorded 100% mortality after 132 hours starvation with first stage zoea. From this it could be concluded that higher DHA and Σ omega-3 fatty acid levels in the zoea improves larval "fitness".

On examining the relationships between the salinity LD_{50}s and the broodstock and egg parameters, only female size showed a significant correlation to larval survival, with larger crabs producing poorer quality larvae. Egg size at extrusion was, however, not correlated ($p \geq 0.05$) to female size. Although none of the egg and zoeal fatty acid profiles were correlated to the salinity LD_{50} values, the EPA and the Σ omega-3 content of the eggs were significantly correlated to broodstock size, with the amounts of these fatty acids decreasing with increasing broodstock size. This may be the explanation for the observation that larval survival is significantly correlated with broodstock size, as these fatty acids in particular are known to influence larval quality (Lavens *et al.* 1991).

Similar results were found in the formalin stress tests. Formalin LD_{50} values decreased as the size of broodstock and the time spent in captivity increased. Significant correlations were also found between the formalin LD_{50} values and the EPA and Σ omega-3 content in newly extruded eggs. In all cases, larval survival in the formalin test decreased as the amounts of EPA and Σ omega-3 fatty acids decreased in the eggs. These findings also suggest that EPA and the Σ omega-3 fatty acids are indicators of egg quality. Results obtained in the ammonia stress tests showed no correlation between any of the recorded broodstock and egg parameters.

Considering the four stress tests as a retrospective measure of egg quality, it is clear that there is a high degree of variability within tests and between the respective tests. However, despite the variability certain parameters do seem to be related to egg quality, manifested by higher survival of the zoea. The starvation and formalin tests suggest that higher EPA, DHA and Σ omega-3 levels in the eggs result in higher larval survival and are therefore indicators of good egg quality. This finding is important, as EPA, ARA and Σ omega-3 fatty acids in the eggs appear to be set aside during embryonic development for future use during larval development. Female size was identified as a possible determinant of egg quality in both the salinity and formalin stress tests with weaker larvae originating from eggs spawned by larger females. This finding is further supported by the observation that the amount of EPA and Σ omega-3 found in eggs decreases as broodstock size increases.

Conclusions

Egg colour had no effect on hatch synchronicity or hatch success rate. Egg colour was also independent of broodstock size or the time females spent in captivity prior to spawning. Moreover, egg quality, measured in terms of zoeal survival in the stress tests, was also not correlated to egg colour. Hatch synchronicity did not affect the hatch success rate and was independent of crab size, egg size at extrusion and at time of hatching. Hatch synchronicity did not have an effect on zoeal survival in the stress tests and therefore cannot serve as an indicator of egg quality. Delayed hatching, (poor synchronicity) may, however, affect overall zoeal survival by inducing starvation stress in those zoea that hatched early. The lipid profiles of newly extruded eggs had no effect on the hatch success rate or hatch synchronicity. The levels of EPA and Σ omega-3 in newly extruded eggs were affected by broodstock size, with larger crabs producing eggs with lower levels of these fatty acids. The lipid profile of newly hatched zoea was not related to the size of the female crabs or the time the broodstock spent in captivity, but EPA levels were related to the size of extruded eggs. Larger crabs tended to produce poorer quality eggs. The stress tests also identified EPA and Σ omega-3 levels in the eggs as the most likely indicators of egg quality, as survival of newly hatched zoea decreased as the levels of these fatty acids in the eggs decreased.

Chapter 5

General discussion and summary

At present mud crab aquaculture is dependent on wild caught seed, as larval rearing protocols, under captive conditions, have not yet been perfected. Numerous studies have been undertaken in an attempt to rear mud crab larvae from egg to first crab stage but all have been characterised by poor survival and inconsistent results (Ong 1966, DuPlessis 1971, Mann *et al.* 1999). The aims of the present study were to gain a better understanding of the spawning and egg characteristics and possible determinants of egg quality in South Africa of *Scylla serrata*.

Wild broodstock were caught successfully year round in estuaries in northern KwaZulu-Natal. The animals acclimated well to captive conditions and spawning usually occurred within 38 days, once introduced into the maturation system. Spawning also took place year round irrespective of what time of the year the females were caught in the wild. From these observations it was concluded that seed supply is not a limiting factor for the development of aquaculture protocols for *S. serrata* in South Africa.

Egg attachment to the pleopods irrespective of crab size or egg colour was good. However, eggs were dropped in all batches incubated by the females. On average, egg batches that were infected with fungus or nematode worms lost ± 13 % of the total egg mass, while uninfected batches only lost ± 3.5 %. The majority of the dropped eggs in infected egg batches were, however, viable and were more than likely removed from the egg mass through the grooming actions of the female. In the uninfected batches the dropped eggs were also viable and most likely dropped due to abrasion against the tank bottom (Mathews and Reynolds 1995) and through limited attachment space as the egg diameters increased during development (Lardies and Wehrtmann 1996).

Although *in-vitro* incubation of crustacean eggs has been successful in certain species (De Caluwe *et al.* 1995_b, Perez *et al.* 1999), it proved to be an ineffective method for

incubating *Scylla serrata* eggs in this study (Chapter 3). Hatch success rates in all trials were poor and much lower than naturally incubated eggs. Artificial incubation has been attempted in other species partly to have greater control over the environmental factors during incubation (Perez *et al.* 1999) and for experimental research to obtain a more precise assessment of egg development. The index of embryonic development as developed and used in this study proved to be an effective means with which to predict the date of hatching and allowed for the timely preparation of live foods and larval rearing tanks.

Fecundities and hatch success rates in this study were comparable to other studies conducted on *S. serrata* in Australia (Mann *et al.* 1999). Hatch success rate in this study was not affected by broodstock size nor was it affected by egg colour or the hatch synchronicity of the different egg batches (Chapter 3). Egg diameter at extrusion showed no relationship to broodstock size or the hatch success rate of the different egg batches. This finding supports the work presented by Hines (1986) who found that there was no relationship between egg size and female size in the brachyurans except in the family Majidae.

A great deal of anecdotal evidence exists concerning the major determinants of egg quality in fish (Brooks *et al.* 1997). Factors such as the age of the broodfish (Brooks *et al.* 1997), diet of the broodfish (Watanabe *et al.* 1991, Harel *et al.* 1994), hormonal status of the broodfish (Campbell *et al.* 1994), bacterial colonisation of the eggs (Hudson and Lester 1994) and genetics (Lam 1994) have all been implicated in egg quality studies. However, our current knowledge on the processes affecting egg quality is however limited (Brooks *et al.* 1997) and also compounded by the fact that egg quality has been found to show considerable variability both within species and over time (Kjørsvik *et al.* 1990).

Previous studies on *Scylla* species have identified egg colour, poor egg attachment and hatch synchronicity as possible determinants of egg quality (Ong 1966, DuPlessis 1971, David Mann, Bribie Island Aquaculture Research Centre, Queensland, Australia. pers.

comm.). During this study both egg colour and hatch synchronicity varied greatly (Chapter 4). Egg colour ranged from pale yellow to orange-red while hatch synchronicity (the time taken for eggs to hatch) ranged from less than 1 hour to 18 hours. However, there was no relationship between egg colour and hatch synchronicity (Chapter 4). Moreover, there was no relationship between egg colour, hatch synchronicity and hatch success rate, indicating that neither of these two egg parameters affected egg quality. This conclusion was validated by the stress tests, which showed no relationships between any of the stressors, egg colour, hatch synchronicity or hatch success rate.

The effect of supplementing crustacean broodstock diets with EPA on larval quality has been extensively studied in the last few years (Xu *et al.* 1994, Kontara *et al.* 1995, Glencross *et al.* 2002). During this study broodstock diet was not supplemented with essential fatty acids but the fatty acid profiles of newly extruded eggs and newly hatched zoea were analysed. These fatty acid profiles were then compared to the various broodstock and egg parameters to assess if they could be related to egg quality (Chapter 4). Findings in this study suggest that the levels of DHA, EPA and the Σ omega-3 fatty acids are important determinants of egg quality in *S. serrata*.

In some cases the relationships between egg quality and the respective fatty acid content in the eggs were indirect. For example, in the salinity stress test survival of newly hatched zoea was negatively correlated to broodstock size but showed no relationship with any of the fatty acids in the eggs and zoea. However, EPA content in eggs was related to broodstock size and, therefore, indirectly may have affected egg quality. This suggestion is backed up by the fact that results obtained in the formalin stress test also identified EPA and the Σ omega-3 fatty acids as factors affecting egg quality.

In this study the following factors have been identified as possible determinants of egg quality, viz. broodstock size, the time females spent in captivity prior to spawning and the DHA, ARA, EPA and Σ omega-3 fatty acid content of the eggs (Chapter 4). This suggests that further research should focus on *S. serrata* broodstock nutrition and husbandry. According to the available literature, no attempt has been made to make use

of crabs that have matured and copulated in captivity for hatchery purposes. This practice would reduce many of the unknown factors encountered when using wild caught broodstock females. For example, the spawning history of the broodstock would be known, dietary composition and intake during maturation can be controlled and the time between mating (deposition of the spermataphore in the female) and fertilization would be known. All of these factors have been implicated as possible causes for poor hatch success rates of eggs in *S. serrata* in past studies (Ong 1966, DuPlessis 1971).

The role of broodstock nutrition in egg and larval quality has been extensively studied in a variety of fish and crustacean species (Xu *et al.* 1994, Peleteiro *et al.* 1995, Morehead *et al.* 2001). As yet, however, the nutritional requirements of *S. serrata* and the fatty acid compositions needed to ensure optimum inclusion of fatty acids in eggs has been poorly studied. Only recently have studies been undertaken on *S. serrata* to assess the relationships between broodstock diet and reproductive and larval performance (Millamena and Quinitio 2000). However, even in these studies broodstock were obtained from the wild and their prior nutritional status and level of ovarian maturation was unknown.

In conclusion, this study has highlighted the need for controlled and standardised broodstock husbandry practices. Capture of broodstock and spawning of wild caught broodstock in captivity is reliable in terms of seed supply and by no means a bottleneck in the development of aquaculture protocols for *Scylla serrata* in South Africa. However, the high degree of variability between different egg batches has highlighted the need to try and minimise the number of unknown factors. Theoretically this could be achieved by capturing late juveniles and rearing these to maturity in captivity under controlled husbandry and nutritional conditions. This approach may lead to speedier resolutions surrounding the questions of egg quality in *Scylla serrata.*

Chapter 6

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