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Hatchery Techniques for Chinese mitten crab *Eriocheir sinensis*

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences (Aquaculture)

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Broedhuistechnieken voor de Chinese wolhandkrab *Eriocheir sinensis*

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Eriocheir sinensis adult / microscopic view of *Eriocheir sinensis* megalopa

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I wish to dedicate this thesis to my family, my husband Xiaolei and my son Star, who have sacrificed a lot during my four years intensive study. This thesis is a present for them.

Table of contents

List of abbreviations

ARA	Arachidonic acid
ANOVA	Analysis of variance
D	Dark
DAH	Day after hatch
DHA	Docosahexaenoic acid
DW	Dry weight
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl easters
FAO	Food and agriculture organization
GSI	Gonad somatic index
HSI	Hepatopancreas somatic index
HUFA	Highly unsaturated fatty acid
L	Light
LC_{50}	Lowest concentration at which 50% mortality is recorded
ICES	International council for the exploration of the sea
LSI	Larval stage index
M	Megalopa
MUFA	Mono-unsaturated fatty acid
PUFA	Poly-unsaturated fatty acid
PVC	Polyvinyl chloride
SFA	Saturated fatty acid
WW	Wet weight
Z1-Z5	Zoeal stage 1-5

Chapter I

General introduction

Chapter I
General introduction

World aquaculture production has grown significantly during the past two decades. Total aquaculture production in 2005 reached 47.8 million mt, accounting for 33.7% of total fishery production (147 million mt). China remains by far the largest producer, with a production of 30.6 million mt in 2004, accounting for 69.7% of the total quantity and 51.2% of the total value of world aquaculture production (FAO, 2007).

Among the major groups of aquaculture species, the production of crustaceans has shown the strongest growth in the new century. It reached 3.69 million mt accounting for 6.56% of total world aquaculture production in 2004. In addition, crustaceans are amongst the most highly priced seafood, valued at 14.4 billion US$, which constitutes 20.4% of the total value of world aquaculture in 2004 (FAO, 2007).

Mitten crab (*Eriocheir sinensis*) is one of the few crustacean species that is being produced on an industrial scale. It is considered a luxury aquatic food due to its delicate flavor, and it therefore reaches a high market value. Mitten crab is also considered very nutritious. It contains high quality proteins with a well-balanced essential amino acid composition and is a good source of trace minerals. Unlike penaeid shrimp and freshwater prawn (*Marcobrachium* spp.) that are produced in large parts of the tropics and sub-tropics, mitten crab is only being cultured in China. Because culture is limited to China, mitten crab farming has attracted very little interest of the international scientific community. Nevertheless, the large production figures and the huge demand by the vast Chinese population (one fifth of the global population) largely justify research on the culture of this species.

Before the 1980s, aquaculture production of mitten crab relied on the provision of megalopa (postlarvae stage before becoming juvenile) caught in the wild and their

restocking in lakes. However, since the 1980s, the natural supply of megalopa and adult crab decreased dramatically due to the construction of irrigation systems, overfishing and water pollution. The decreasing natural recruitment stimulated the development of controlled production of seed to stock ponds.

Crucial breakthroughs in seed production and larval rearing techniques of mitten crab have been achieved ever since the early 1980s. This was further triggered when penaeid shrimp culture in China and South-east Asia collapsed due to severe bacteria and virus outbreaks in the early 1990s. From then on, mitten crab farming expanded tremendously. The recent annual aquaculture production of mitten crab in China reached about 500,000 mt, valued at 2.2 billion US$ in 2005 (FAO, 2007).

The main obstacle for mitten crab hatchery production is the variable and usually low larval survival. Several factors contribute to this problem: inferior broodstock quality due to genetic degeneration and lack of knowledge on broodstock nutrition; low molting synchrony and consequently significant cannibalism (which occurs particularly during metamorphosis of Z5 to megalopa) as a result of suboptimal larval feeding regimes and unbalanced and/or deficient larval nutrition; disease outbreaks caused by bacterial proliferation and an unstable microbial environment in the intensive larval rearing system. Research on the above issues should lead to improvement of mitten crab hatchery techniques, and thus enhance and sustain the mitten crab hatchery production in terms of larval production and larval quality.

Obviously, this thesis could not deal with all the above topics. The general objective of this thesis was to clarify the population structure of natural mitten crab stocks in China and to improve the broodstock reproductive performance, offspring quality and larval performance of mitten crab through the improvement of their nutritional status and feeding regime. The thesis outline can be summarized as follows:

Chapter I (*General introduction*) draws an outline covering the main points of this thesis.

Chapter II (*Literature review*) gives an overview of the natural resources and aquaculture activities of mitten crab in China to date; and reviews the techniques for broodstock maturation, spawning and larviculture. Although breakthroughs in the development of hatchery techniques have been achieved in the early 1980s, many of these techniques have only been published as brief description of local farmer's experiences and most of them are written in Chinese. This chapter includes both published and unpublished data, combined with personnel comments, aiming to provide general information that is essential for promoting hatchery techniques of mitten crab.

Chapter III (*Population genetics*) evaluates the genetic structure of native mitten crab populations and draws a general picture of the worldwide migration and evolution of mitten crab. In its native range in China, mitten crabs are mainly distributed in the Liaohe, Yangtze and Ou River basins and form different geographical populations due to the long term geographical and ecological isolation. Most demanded is the crab originating from the Yangtze River due to their better growth rates and delicate flavor, and are therefore most frequently used in local hatcheries and farms. However, as a drawback of the booming aquaculture activity, the shortage of broodstock and juveniles has resulted in frequent translocations of animals among the different geographical populations, which endangers the integrity of the genetic diversity of this species. Also, mitten crab has been repeatedly introduced from native habitat into Europe and America via ballast water over the past 100 years, where it has become one of the most notorious aquatic invaders. Therefore a better understanding of the natural population structure is crucial for the conservation of this over-exploited species and investigation of invasion process and evolutionary implication of mitten crab.

Chapter IV (*Broodstock nutrition*) consists of three parts:

- *Effects of dietary soybean lecithin on reproductive performance of Chinese mitten crab* Eriocheir sinensis *broodstock* (Section I)
- *Effects of dietary HUFA on tissue fatty acid composition and reproductive performance of Chinese mitten crab* Eriocheir sinensis *broodstock* (Section II)
- *Effect of diets on reproductive performance and HUFA composition of Chinese*

mitten crab Eriocheir sinensis *broodstock during second spawning* (Section III) *

Although the mitten crab has been domesticated and its reproductive cycle has been closed already two decades ago, the unstable yields in terms of larval production and larval quality is still a major constraint in many hatcheries, which, at least partly, is believed to relate to incomplete and unbalanced broodstock diets. Phospholipids and highly unsaturated fatty acids (HUFA) are considered as important nutrients for crustacean broodstock maturation and hence are essential in broodstock diets. Section I and Section II investigate the effect of increasing dietary soybean lecithin and HUFA levels on reproductive performance of broodstock in terms of ovary maturation, fecundity, egg production and egg quality using semi-purified compound feeds.

Mitten crabs are capable of releasing up to three batches of eggs from one single mating. Utilization of subsequent spawnings therefore would extend the reproduction window and increase larvae production efficiency. However, a substantial reduction of broodstock survival, egg production and larval quality are generally observed in subsequent spawnings. Nutritional requirements are believed to be higher during re-maturation of depleted ovary and therefore nutritional effects are expected to be more evident. Section III briefly describes the effect of three natural diets (clam, sandworm and trash fish) and one artificial diet on the reproductive performance and larval quality of second spawning mitten crab broodstock.

In **Chapter V (*Larval nutrition*)** different aspects of larval feeding and larval nutrition were studies:

- *Ingestion of rotifers and Artemia nauplii by Chinese mitten crab* Eriocheir sinensis *zoea larvae* (Section I)
- *Effect of feeding scheme and prey density on survival and development of Chinese mitten crab* Eriocheir sinensis *zoea larvae* (Section II)

* This experiment was performed in the early part of the thesis work

- *Effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab* Eriocheir sinensis *zoea larvae* (Section III)

In the hatchery phase, mitten crab larvae are mostly cultured on a sequence of microalgae, rotifers and *Artemia* according to the larval stages. Despite a long tradition in larvae culture, feeding of the larvae in hatcheries depends on the experience of the farmers as very little research has been performed. Aiming to improve larval survival and growth, and to minimize food waste and pollution of culture water, Section I determines the daily *Artemia* ingestion rate based on individual zoea larvae (first five larval stages of mitten crab, called zoea 1-5) with or without rotifer co-feeding; Section II further investigates the optimal time to introduce *Artemia* into the diet as a supplement to rotifers and the optimal densities of rotifers and *Artemia* through the entire larval rearing period.

Rotifers and *Artemia,* often lack n-3 HUFA, such as EPA (in rotifers) and DHA (both in rotifers and *Artemia*), which are recognized as essential fatty acids for marine crustaceans larvae and postlarvae. Although normal growth and development are obtained in mitten crab larval rearing, high mortality is frequently occurring when larvae are stressed, especially during the molt from Z1 to Z2 and metamorphosis from Z5 to megalopa. Section III determines whether boosting rotifers and *Artemia* with different levels of n-3 HUFA and DHA/EPA ratios affects growth, survival, metamorphosis and osmotic stress tolerance of mitten crab zoea larvae.

Chapter VI (*General discussion*) reiterates and discusses the overall results of the experiments conducted in this thesis. Based on the discussion, the general conclusions are drawn and prospective research topics are proposed.

Chapter VII (*References*) contains all the bibliographic citations mentioned in this thesis.

Chapter II

Literature review

Chapter II

Hatchery techniques for Chinese mitten crab *Eriocheir sinensis*

1. General information

Natural distribution

The Chinese mitten crab, *Eriocheir sinensis* (H. Milne-Edward, 1835) (Crustacea: Decapoda: Grapsidae) originates from the East Asia, with a native distribution from the eastern pacific coast of China, 24^{o}N northwards to the Korean Peninsula，42-43^{o}N; from 124 ^{o}E westwards to 112 ^{o}E (Gu and Zhao, 2001). However, with the development of mitten crab culture in recent years, the species was spread almost throughout the country. Overall, three major populations are recognized according to the river estuaries where they live: the Liaohe, Yangtze and Ou River. The Yangtze River basin is the central area of their distribution in China. Although a number of studies have been conducted to distinguish the different geographical populations through the analysis of morphological, biochemical and molecular variations (Li and Li, 1996; Xu et al., 1997; Li and Li, 1999; Li and Zou, 1999; Zhao and Li, 1999; Zhou and Gao, 1999; Wang and Li, 2002), the structure of native mitten crab stocks still remains unclear in terms of population genetic structure.

Out of its native distribution range, the mitten crab proved a successful aquatic invader. As an inhabitant of temperate environments, this species has easily adapted to similar conditions in Europe and it was in 1912 that the first specimen was recorded from the River Aller in Germany (Panning, 1939). From then, mitten crab flourished, spreading throughout Europe, from Scandinavia to the Atlantic coasts of France and Portugal, and England (Herborg et al., 2003). It has more recently become established on the west coast of the US in the San Francisco Bay (Nepszy and Leach, 1973; Rudnick et al., 2003), the Chesapeake Bay and the mid-coast of North America (Ruiz et al., 2006). This

invasive species causes considerable economic and ecological damage due to its burrowing activity as well as predation on native invertebrates and commercial fish (Rudnick et al., 2005). The most probable mechanism of introduction was accidental release via ship's ballast water (Cohen and Carlton, 1997).

Feeding behavior

Mitten crab feed on a variety of food items. Larvae eat primarily phytoplankton (diatoms, dinoflagellates, blue-green algae and green algae), rotifers, copepods and cladocerans. Juveniles, which are more mobile, feed on macrophytes, algae, oligochaetes and detritus (Jin et al., 2001). Larger adult crabs eat widely diversified diets including marcophytes, algae, arthropods, oligochaetes, gastropods, mollusks, prawns, fishes, submerged aquatic weeds, pumpkins, maize and detritus (Jin et al., 2003), and are best described as opportunistic omnivores.

Morphology & Life cycle

The main identifying features of the mitten crab are the dense patches of hairs on the white-tipped claws of larger juveniles and adults (Fig. 1). Its scientific name *Eriocheir sinensis* derived from Greek, means "wool hand, the Chinese". The shell (carapace) is quite square in outline, narrowing towards the front. It has four spines on either side, and reaches a width of approximate 8 cm. The legs of the adult crab are generally more than twice as long as the width of the carapace. As a catadromous species, the 2-year old adults (often called green crab) migrate downstream to reproduce in late autumn. The migration duration is approximate one month and the migration rate is about 24-40 km per day (Zhang and Li, 2002). Mating occurs in the brackish water of estuaries. The females carry approximate 1 million eggs over winter until hatching. After a 1-2 month period as planktonic larvae, the small juvenile crabs settle out in brackish water in late spring, then migrate upstream into freshwater often for considerable distances, to grow to adulthood (Fig. 2). The upstream migration rate is much slower than that of the downstream one, namely 8-12 km per day (Du, 2004). Generally, mitten crab molts 18

times throughout its life.

10 cm

Fig. 1. Dorsal view of adult male Chinese mitten crab *Eriocheir sinensis*

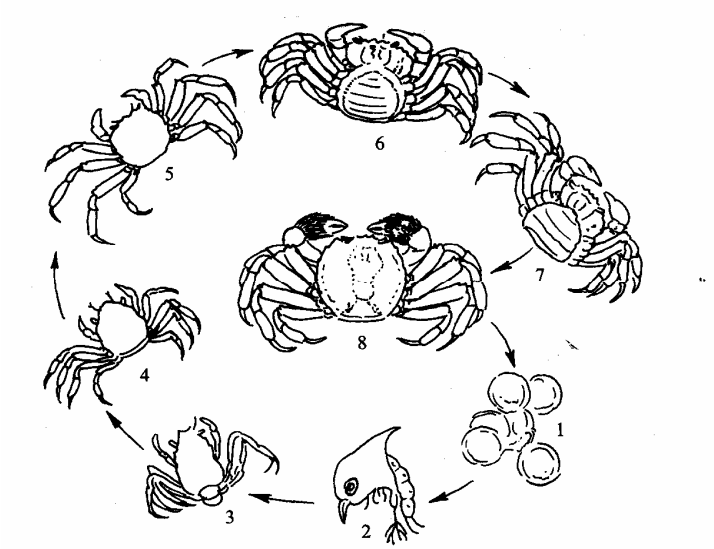

Fig. 2. Life cycle of Chinese mitten crab *Eriocheir sinensis* (After Xu and He, 1994)
1) Eggs; 2) Zoea; 3) Megalopa; 4) First juvenile stage; 5) Soybean-size crab; 6) Coin-size crab; 7) Yellow crab; 8) Green crab (broodstock)

Nutritional value

Mitten crabs are considered as a most nutritious and delicious crustacean by the Chinese consumer. The history of using mitten crab as food dates back more than 1000 years. Analysis of the nutritional quality of mitten crab showed that meat and edible viscera account for 24.2% and 9.2% of the total body weight. Its crude protein (18.9% of meat weight) is considered a high quality protein with a well-balanced essential amino acids composition. It contains high levels of glutamic acid (151 mg g^{-1}), aspartic acid (99 mg g^{-1}), arginine (99 mg g^{-1}), lysine (81 mg g^{-1}) and leucine (77 mg g^{-1}). Mitten crabs are also an excellent source of minerals, particularly zinc, iron, copper and phosphorus (Chen et al., 2007).

Decline of natural populations & Rise of crab farming

From the mid 1960s to mid 1980s, aquaculture production of mitten crab relied on the provision of wild megalopa and the restocking of captured megalopa in lakes. The natural yield of megalopa in the Yangtze River estuary in the 1970s and early 1980s fluctuated between 1 mt to 20 mt. However, it dropped to less than 10 kg in the late 1980s and the 1990s (Yu et al., 1999) (Fig. 3). The same trend occurred in the fishery landings of adult crabs, which were mostly above 20 mt before 1985, and then dropped to less than 15 mt afterwards (Fig. 4). The dramatic decrease of the natural yield of megalopa and adult crabs were mainly caused by obstructions of their reproductive migration due to the construction of dams, overfishing and water pollution (Zhao, 2000; Shi et al., 2002). The variable and decreasing natural recruitment, however, has greatly stimulated the development of controlled production of seed to stock ponds.

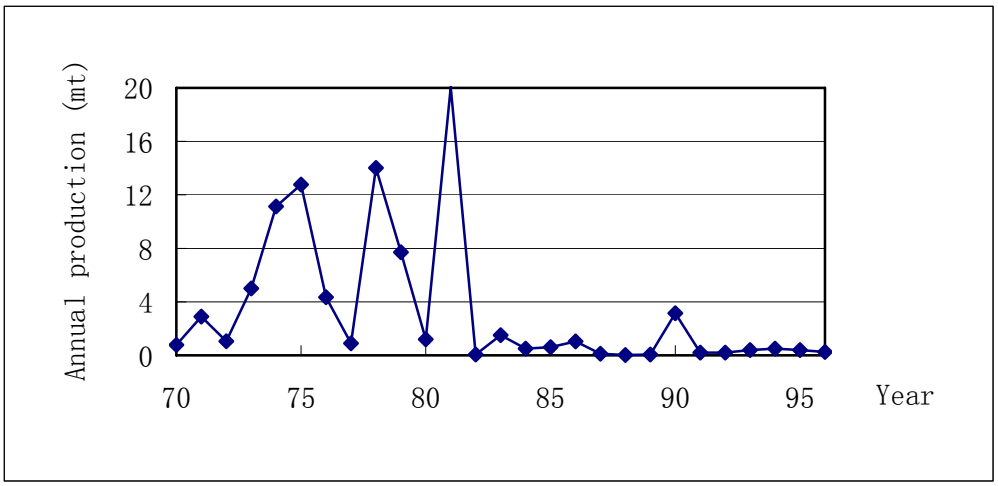

Fig. 3. Natural yield of *Eriocheir sinensis* megalopa in the Yangtze River estuary (after Yu et al., 1999)

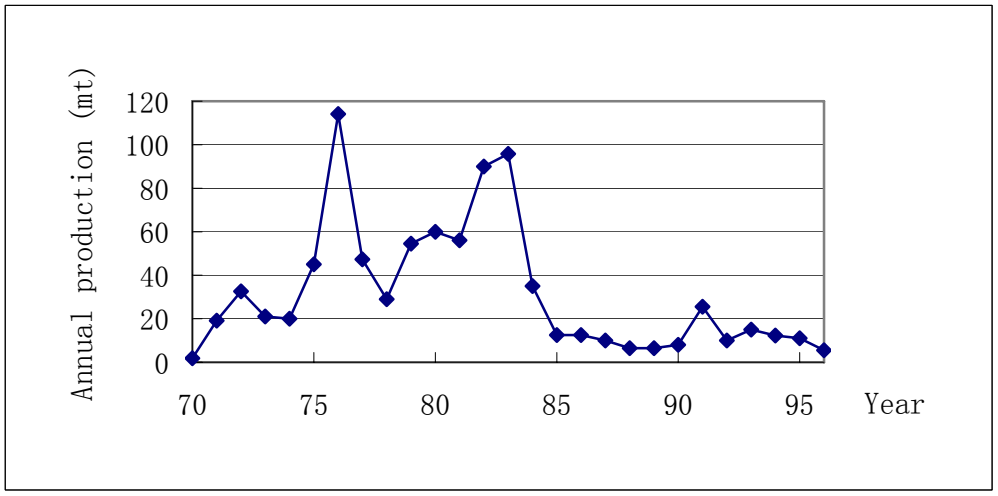

Fig. 4. Fishery landings of *Eriocheir sinensis* adults in the Yangtze River estuary (after Yu et al., 1999)

A breakthrough in hatchery techniques for mitten crab was achieved in the 1980s by using artificial seawater (Zhao, 1980) and natural seawater (Xu and He, 1994). Since then, mitten crab culture increased remarkably, especially when penaeid shrimp culture in China and other Asian countries was hit by severe virus outbreaks in the early 1990s. The annual yield of market-size mitten crabs increased from 17,500 mt in 1993 to 500,000 mt in 2005 (Fig. 5). Meanwhile, the total yield of megalopa produced in local

mitten crab hatcheries doubled from 277 mt in 2001 to 522 mt in 2003 within only three years time (China Fisheries Yearbook, 2004).

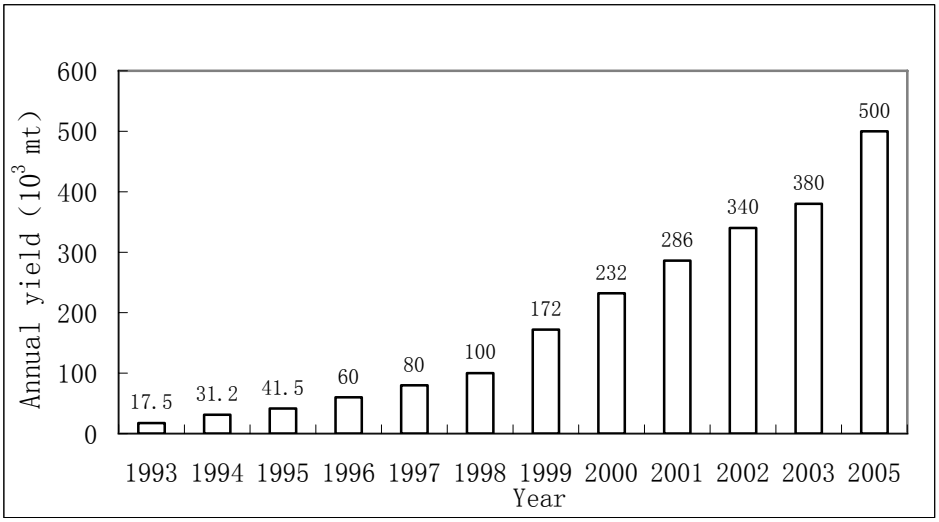

Fig. 5. Annual aquaculture yield of *Eriocheir sinensis* in China

2. Culture

Mitten crab culture can roughly be divided in three phases: broodstock management and megalopa production (hatchery phase), coin-size seed crab culture and growout. In this chapter, the techniques on broodstock management and larval rearing are discussed.

2.1 Broodstock

Origin

Mitten crab is indigenous in the main river systems in northern and central China. Populations from the Yangtze River (YR) and Liaohe River (LR) are thought to be the most productive and used as the primary broodstock source for aquaculture. Apart from the morphological, biochemical and molecular differentiation, there are some differences on growth performance between the two populations (Li Y.S. et al., 2000; Li et al., 2001). YR is the favored broodstock resource due to its better growth performance and more delicate taste.

Mature crabs are most commonly retrieved from production ponds or natural lakes, where commercial culture is practiced. Only few breeders can be caught from the wild due to the low level of wild stock during the last decade. The life cycle can be closed in minimum 9 months (for LR population) up to 2 years.

After the puberty (=ultimate) molt, the carapace color of mitten crabs (usually 2 years old) changes from yellow to green, indicating the maturity of the crabs. Good breeders are identified by the following features: green shell, U-shaped abdomen fringed with setae, healthy and vigorous, intact claws and walking legs, no attached parasites or other organisms. It is generally observed that larger breeders produce larger quantities of eggs, but with higher mortality during egg incubation, especially when reaching the stage of egg hatching, therefore animals with a body weight of 120-150 g for females and 150-200 g for males are usually preferred as breeders in hatcheries. When kept moist broodstock can be transported to the maturation system without water for long distances.

Broodstock management

In commercial hatcheries, the selected males and females are maintained separately in freshwater. Before mating, the salinity of the water needs to be elevated in order to achieve the final maturation of both female and male crabs (Zhang and Li, 2002). Mitten crabs mate and spawn at a water salinity of 8-33 g L^{-1}, with an optimum at 17-20 g L^{-1}. Water temperature ranges from 9 to 13°C. The ratio of female and male is 2:1 to 3:1. One day after copulation, berried females can usually be observed. In order to avoid multiple mating, the berried females are removed daily from the mating tank and the number of male crab is adjusted according to the number of non-berried female left in the tank. The spawning percentage can be up to 70-90% within a week.

Mating is done either in late autumn or early spring when water temperature is low enough. In the first case, the berried females carry the eggs in seawater over winter, which is generally considered as the best way to obtain a high spawning rate and good

egg quality. Mating may also be delayed until the following spring (March) in order to avoid maintaining ovigerous crabs over winter, but this gives a risk of producing zoea 1 (Z1) with lower quality. In practice, broodstock is maintained in either outdoor earthen ponds or indoor concrete tanks. Substrate, such as mud, fine sand or a mixture of both, is generally spread over the bottom of indoor concrete tanks in a layer of 5-10 cm, and cleaned regularly with an upwelling water system or a flow-through water exchange. The crabs dig a crater in the substrate over which the abdominal flap is extended into which the eggs are extruded. The pleopods then create a current that assists the attachment of eggs to the setae. Absence of a spawning substrate usually results in poor egg attachment and potential loss of the batch. In order to reduce stress and prevent cannibalism, stocking density in the maturation systems is usually kept not higher than 3-5 crab m^{-2} in outdoor ponds, but broodstock can be stocked up to 10 crab m^{-2} in indoor concrete tanks, where good water recirculation is provided.

Mitten crabs inhabit turbid estuarine water in nature; therefore light is usually reduced by covering the tanks in the indoor conditions. The broodstock is normally fed fresh or frozen food with an adequate nutritional value, including shrimp, squid, fish, mussels, snails and annelid worms. Feed is usually supplied *ad libitum*, but when rationed, crabs are fed at daily rates ranging from 3-5% to 20% of their body weight depending on the water temperature. Due to their nocturnal feeding habit, food is normally provided in the late afternoon at 16-18 hrs.

Spawning

In nature, berried crabs take 6-7 months to develop fertilized eggs to zoea larvae, of which the eggs spend 3 months in the gastrulae stage under lower temperature (Zhou, 1989). Therefore the window of seed production is very limited, i.e. only in the period of April to May in the Yangtze River estuary (Zhang et al., 2002). In the hatchery, the spawning of captive mitten crabs, however, can be modulated through manipulation of the incubation temperature. The egg hatching can be advanced to December and January through a steady increase of the incubation temperature straight after spawning.

On the other hand, by stocking the berried crabs under low temperatures (6-8°C), at which the embryo development is infinitely delayed in the gastrulae stage, the egg hatching can be postponed to June without negative impact on the survival of broodstock and larval quality（Wang and Zhao, 2000; Sui et al., 2007). Nowadays these manipulations are usually applied on site, to extend the reproduction window and thus improve the effectiveness of facility utilization in the hatchery.

Mitten crab is capable of spawning up to three times from a single mating. Mated females carry spermatophores without the need for another mating. Fecundity is normally about 1 million eggs for the first spawn. Second maturation of the ovary in captive crabs occurs about a month after the first spawn, which is much shorter than for first maturation (3-4 months). A substantial reduction of broodstock survival, egg production and larval quality is however generally observed with subsequent spawns (Nan et al., 2006). Few hatcheries make use of re-matured broodstock due to the fact that egg supply is not a limiting factor in mitten crab hatcheries.

Broodstock maturation

The morphology and ultrastructure of the mature egg and vitellogenesis of *Eriocheir sinensis* during first maturation process were studied by means of electron microscopy and histochemical methods (Du et al., 1995; Cheng et al., 1999a; Du et al., 1999; Yang et al., 2007). The oogenesis of *Eriocheir sinensis* can be divided into four or five phases, including the oogonium phase, the minor growth phase, the major growth phase, (pre-maturation phase) and the maturation phase (Xue et al., 1987; Gu and He, 1997).

Based on the development of the germ cells and the size and color of the ovary, the ovary development can be divided into five to seven stages (I to V/VI/VII) (Xue et al., 1987; Gu and He, 1997; Wen et al., 2001), which are conveniently grouped into three periods: lag phase (May to July), log phase (August to November) and stationary phase (December to April). The ovary maturation seems to vary between the geographical locations, generally associated with extremes in seasonal water temperatures or

salinities (Gu and He, 1997). Moreover the final ovarian maturation can only be reached when animals are kept in seawater and in the presence of males. When females stay in freshwater for all their life, their ovaries degenerate and are absorbed by the follicle cells. Unilateral eyestalk ablation promotes ecdysis, growth and ovary development in early grow-out stages, but unlike in other decapod species (e.g. *Penaeus monodon, Penaeus penicillatus and Macrobrachium nipponense*), it has no effect on ovary maturation and spawning of *Eriocheir sinensis* broodstock (Gu and He, 1991).

In crustaceans, the hepatopancreas is regarded as a major lipid storage organ (Harrison, 1990). The ultrastructural studies revealed that the hepatic lipid in *Eriocheir sinensis* was principally stored in hepatopancreatic R (receptive) cells in the form of lipid droplets (Cheng et al., 2000a; Jiang et al., 2001). During the ovary development, the hepatopancreas somatic index (HSI) decreases remarkably (from 6.56 to 3.77 of wet body weight) with a concomitant drop of lipid content (from 34.37 % to 28.13% of wet hepatopancreas weight) (Cheng et al., 1998a). However, the lipid composition remains constant, consisting of 80% neutral lipids (NL, mainly triacyglycerol (TG)) and 10-20% polar lipids (PL, mainly phosphatidylcholine (PC) and phosphatidylethanolamine (PE)). On the other hand, 18:1n-9, 16:0 and 16:1n-7 are the major fatty acids present in the hepatopancreas. The mono-unsaturated fatty acid (MUFA) content is higher in the hepatopancreas of *Eriocheir sinensis*; but the saturated fatty acid (SFA), poly-unsaturated fatty acid (PUFA), n-3 and n-6 highly unsaturated fatty acid (HUFA) content, especially 20:5n-3 and 20:6n-3, are lower compared to marine crustacean species, e.g. *Penaeus monodon* and *Penaeus chinensis* (Ying et al., 2004).

Like in other crustaceans, the hepatic lipids are mobilized to the ovaries via the hemolymph in the process of ovary maturation of *Eriocheir sinensis* (Cheng et al., 1998a; Wen et al., 2001). The ovarian lipid content in wet ovarian weight increases steadily from stage II (5.4%) to stage IV (19.1%) and decreases to the lowest level after spawning (stage V, 6.6%); whilst the hepatic lipid content increases with maturity of the ovary, reaching a maximum at stage III (29.9%), and decreases during the subsequent period to spawning (16.7%). Both TG and PC are responsible for the increase in ovarian

lipid content during maturation. Cheng et al. (1999b) demonstrated that ovarian lipid is mainly stored in lipid droplets and yolk; among which, lipid droplets contain more NL, and yolk contains more PL. The composition of NL, PL and fatty acids in the ovary are similar to the hepatopancreas. The content of short-chain saturated and unsaturated fatty acids in PL are less than in NL, whilst the content of 20:5n-3 and 20:6n-3 in PL is more than that in NL. 16:0, 16:1n-7, 18:1n-9, 18:2n-6, 20:4n-6, 20:5n-3 and 22:6n-3 are the major fatty acids occurring in the broodstock ovary. And the percentage of 20:5n-3 and 20:6n-3 in both NL and PL increases, but 20:4n-6 decreases during the maturation process (Cheng et al., 2000b). Moreover, lower concentrations of PL, 20:5n-3 and 20:6n-3 are observed in the ovary of aborted or un-berried females as compared to berried females, which confirms the importance of PL and HUFA in *Eriocheir sinensis* maturation. In contrast, Ying et al. (2006) indicated that the SFA, MUFA, PUFA levels and the n-3/n-6 ratio in the ovary and hepatopancreas are significantly different. It is also noteworthy that arachidonic acid (20:4n-6) was only found in crabs that lose their eggs, meaning that it might relate to abortion or egg loss.

Tian et al. (2002) reported that, during embryogenesis, protein content (% dry weight) decreases dramatically from 67.76% in fertilized eggs to 44.56% in protozoea, while lipid content increases from 21.32% to 34.07% and water content increases from 52.90% to 71.76%, indicating protein is mainly used as energy source for embryonic development. On the other hand, Cheng et al. (1998b) demonstrated that mainly lipid was used as energy source with constant lipid content during embryonic development. Although part of PL is responsible for the tissue and organ differentiation, PL is also used as a main energy source during the embryonic development. NL (mainly TG) is always a major lipid in the eggs, but only a little is used as energy in the process of embryogenesis; plenty of it is reserved for the newly-hatched zoea larvae.

Broodstock nutrition

A typical feature of poikilotherms is that they are capable of starving for long periods. *Eriocheir sinensis* broodstock would utilize fat as primary and predominant metabolic

substrate, carbohydrate as secondary, and protein as tertiary substrate under both standard and starvation metabolism (Wen et al., 2002a; 2002b). The latter authors furthermore have shown that starvation during ovary maturation does not influence spawning of the broodstock. The effect of starvation on the fecundity, egg quality and hatchability of *Eriocheir sinensis* however needs some further investigation.

Nevertheless, broodstock nutrition has been shown to affect egg quality in crustaceans (Lavens et al., 1991). And the accumulation of essential nutrients within the egg is dependent on the nutrient reserves of the mother and the diet in the period preceeding gonadogenesis (Harrison, 1990; 1997). However, only limited information is available about nutritional requirement of mitten crab broodstock. Ai et al. (2003) demonstrated that supplementing the broodstock diets with vitamin C and vitamin E, alone or in combination (0.5 mg Vit C/100g diet; 0.022 mg Vit E/100g diet; 0.5 mg Vit C + 0.022 mg Vit E/100g diet) improved the reproductive performance, in terms of GSI, fecundity and hatchability of *Eriocheir sinensis*. Through feeding *Eriocheir sinensis* broodstock with semi-purified diets, the importance of dietary HUFA and PL on reproductive performance and offspring quality were demonstrated by Wen et al. (2002c) and Wu et al. (2007b). Furthermore, Wen et al. (2002c) demonstrated that there is a close correlation between the 20:5n-3 content in the egg lipid and fecundity, the 22:6n-3 content and hatchability; and therefore recommend adequate n-3 HUFA provision in broodstock diets. Wu et al. (2007b) concluded that the optimum combined level of PL and HUFA in the diet (% dry diet weight) is 1.6% PL / 2.5% HUFA or 2.3% PL / 2.0% HUFA.

2.2 Larvae culture

Egg incubation

The egg incubation period is strongly temperature dependent. Up to 21°C, a higher incubation temperature leads to a shorter incubation period but smaller-sized larvae. The fertilized eggs develop into zoea larvae within 20 days at temperatures of 15 to 20°C

and a suitable salinity. During the incubation, the broodstock and eggs are vulnerable to infestation by parasitic worms, fungi and ciliates (*Zoothamnium* sp.). The ciliates do not only invade the gill and digestive tract, but also the hepatopancreas, gonads, heart and muscles. Once infected, the crabs suffer from poor appetite, weakness, respiratory difficulties, and eventually die. The infestation of eggs may result in poor embryogenesis or egg loss. The berried broodstock can be bathed in formalin and/or an antifungal agent as a prophylactic treatment before being placed in the incubation tanks, or can be removed from the incubator and treated when infestations occur.

During egg incubation, the crabs provide oxygen to the eggs by moving the abdominal flap. A number of the berried females die during incubation due to the depletion of energy and nutrition, especially when attending to the hatchlings. Several studies have been conducted on *in-vitro* incubation of detached eggs, aiming to utilize this kind of egg resource (Gu et al., 1995; Zhu et al., 1997; Bao et al., 1999). Newly detached eggs (usually within 10 h after spawning) can be incubated and hatched artificially, but hatching rate is often not satisfactory. Fungal infections have so far made this practice ineffective.

Time of egg hatch can be predicted by monitoring development of the embryo under a dissecting microscope. The grey colour and transparency of the eggs, a heart beating rate of 170-200 min^{-1} and well developed eye spots with a purplish patch are all indications of imminent hatching. Before being transferred into the hatching tank, the berried crabs are usually disinfected in a 100-200 g L^{-1} formalin bath for 1-2 h to remove possible attached parasites and fungi. Just before hatching, the water in the hatching tank is usually treated with the chelating agent ethylene diamine tetra acetic acid (EDTA) to reduce the heavy metal load. The berried crabs are normally placed in cages that are suspended inside the hatching tank (no sand provided on the bottom), to which the initial diet for the larvae (usually *Chlorella* sp. and diatoms) had been added in advance already. In order to maintain a better water quality, berried crabs are not fed one or two days before egg hatching. When larval density in the tank reaches a preset level, the cages are removed from the hatching tank and transferred into another

hatching tank, where the females will continue to release larvae. Newly-hatched larvae that sink in the water column are generally considered as poorer quality than those that concentrate at the surface. Since Z1 larvae are highly vulnerable to fungal infections that can spread from the egg envelop, female crabs are normally removed from the hatching tank soon after they have released their larvae.

Larval development

The eggs hatch as zoea larva, which molt six times to complete larval development, i.e. five zoeal stages and one megalopa stage (Fig.6) (Liang et al., 1974; Anger, 1991; Montu et al., 1996). Occasionally, an additional zoea stage (Z6), and in one case, an additional megalopa stage (transitional to the first crab stage) were observed under unfavorable conditions with low salinity (≤15 g L^{-1}) (Anger, 1991). The fertilized eggs develop into juvenile crab within 39-40 days at 11-22°C (Liang et al., 1974). In commercial hatcheries, larval development takes 2-4 days for each of the zoea stages and 5-10 days for the megalopa stage depending on the rearing temperature, food availability and water salinity.

Water quality parameters

Successful development from hatching to metamorphosis occurs only at temperatures ≥12°C. With increasing temperature, both the survival and the range of salinity tolerance increase, whereas development duration decreases exponentially (Anger, 1991). In order to shorten the production cycle and consequently reduce the production cost, in practice the temperature is gradually increases from 21°C at Z1 to 24-25°C at Z5, and then decreases gently to the natural temperatures range at megalopa stage a few days before harvest. Larvae are extremely sensitive to abrupt fluctuations in temperature. Spine injury and low survival are usually observed at Z1 when incubation temperature is abruptly increased (Su and Xu, 1996; Zhu and Li, 1998).

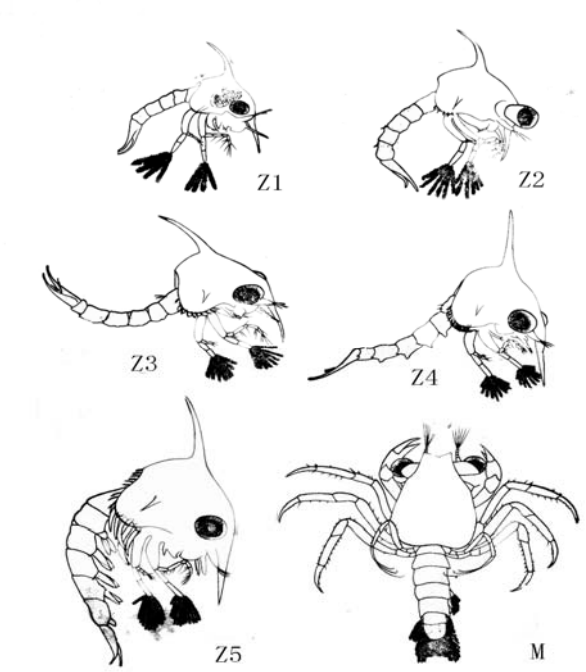

Fig. 6. Zoea and megalopa stage of Chinese mitten crab *Eriocheir sinensis* (after Liang et al., 1974)
Body length (mm): Z1 1.4-1.8; Z2 1.7-1.9; Z3 2.3-2.4; Z4 3.0-3.4; Z5 4.1-4.5; Megalopa 4.2
Average individual body weight (mg): Z1 0.13; Z2 0.27; Z3 0.5; Z4 1.0, Z5 1.8; Megalopa 7.5

Larvae can survive in water with a salinity ranging from 10 to 35 g L^{-1}. The optimal salinity for *Eriocheir sinensis* larvae rearing is about 15-27 g L^{-1}, at which the survival to megalopa can reach more than 10% (Zang et al., 1999). Xiao et al. (2003) reported that 20, 22 and 20 g L^{-1} are the optimal salinity for the Z1-Z2, Z3-Z4 and Z5-megalopa stages, respectively. The larval development duration increases at unfavorably low or high salinities, when mortality also increases. Z1 is very euryhaline, with an optimum in slightly brackish water (25 g L^{-1}). Anger (1991) reported that, during subsequent zoea development, the larvae become increasingly stenohaline, and the optimum salinity shifts to seawater. The megalopa, in contrast, are euryhaline again and developed fastest at lower salinities (15-25 g L^{-1}).

The capacity of osmo-regulation in *Eriocheir sinensis* larvae increases with larval development. Too high and too low water salinities influence the growth and metamorphosis because larvae need to invest more energy in osmo-regulation. The

osmo-regulation of *Eriocheir sinensis* is carried out through Na-K-ATPase present in the gills (Péqueux and Gilles, 1981 and 1988; Gocha et al., 1987). Magnesium acts as an activator of this enzyme, whilst calcium is the key element of calcification for periodical exuviations of crustaceans (Bourget and Crisp, 1975). Therefore the concentration of both elements and the ratio of Mg^{2+}/Ca^{2+} are crucial for the osmo-regulation and metamorphosis of *Eriocheir sinensis* larvae, and must be supplemented through the diets and culture medium. Zhao (1980) reported that using artificial seawater as a rearing medium, the optimal concentration of ions is 144-335 mg L^{-1} for calcium, 200 to 400 mg L^{-1} for potassium, 461-935 mg L^{-1} for magnate and 0.02-0.05 mg L^{-1} for ferric. Similarly, Zang et al. (1998) reported that, 178-340 mg L^{-1} for calcium, 484-816 mg L^{-1} for magnesium and an Mg^{2+}/Ca^{2+} ratio of 2.0-3.0 are optimal when using water from a river estuary as culture medium. On the other hand, the ratio of Na^{+}/K^{+} also has a significant effect on the survival and metamorphosis of mitten crab larvae. Survival increases with decreasing Na^{+}/K^{+}; and the optimal Na^{+}/K^{+} should be equal or lower than natural seawater (27.8).

Compared to fish larvae, *Eriocheir sinensis* zoea larvae are less tolerant to low dissolved oxygen (DO) concentration. A DO level of >4 mg L^{-1} is preferred in zoea larvae rearing. Severe mortality is observed when DO is less than 2 mg L^{-1} (Zhao, 1980). The optimal pH value is 7.5-8.7 for Z1, and for all zoea stages should be less than 9.3 (Zhao and Jin, 2001).

In general, both the larvae and juveniles of *Eriocheir sinensis* are less resistant to ammonia than those of the other crustacean species studied so far (Zhao et al., 1998). The ammonia tolerance increases with larval development from Z3 to megalopa, especially in the stages of Z3-Z4 and Z4-Z5 (no data recorded for Z1 and Z2). The 24h LC_{50} values of unionized ammonia (NH_3) for Z3, Z4, Z5 and megalopa are 1.11, 2.36, 2.77 and 3.18 mg L^{-1}, respectively at pH 8.0, 22 °C and a salinity of 25 g L^{-1}. On the other hand, Jiang et al. (1997) reported the 24h LC_{50} values of NH_3 for Z3, Z4 and Z5 are 1.94, 4.92 and 5.70 mg L^{-1}, respectively, when increasing culture temperature from 23 to 25 °C. Safety concentrations for Z3, Z4 and Z5 are 0.066, 0.196 and 0.189 mg L^{-1}

for NH_3; and 0.713, 1.348 and 2.173 mg L^{-1} for nitrite (NO_2^-). NH_3 is more toxic to *Eriocheir sinensis* larvae, because NH_3 is more lipo-soluble than NO_2^-, and therefore penetrates more easily through the cell membranes.

Xing et al. (1998) reported that the diurnal water parameters in intensive larval rearing tanks fluctuate remarkably. The peak of DO and pH were observed at 15 hrs, whilst the lowest value appeared at 3 hrs; in contrast, NH_4^+-N had a peak value at 3 hrs and the lowest value at 15 hrs; no remarkable change were recorded for COD. Therefore he suggested that water renewal should be applied at 3 hrs in the morning. In practice, water renewal is mostly done after midnight in the hatchery. Several water exchange regimes are employed in intensive larval rearing systems. No water renewal is performed during the first three days of culture (Z1), afterwards daily water renewal is slowly increased from 10% to 20% (Z2 and Z3), to 40-50% (Z4 and Z5), and 100-200 % at the end of the rearing cycle as biomass and feeding levels increase. In some hatcheries, the large tanks are partially filled with green water at stocking Z1 in order to concentrate the larvae, thus reducing the quantity of rotifers and algae required. Clean seawater is added daily, gradually filling up the tanks at Z2 and Z3 stage. Towards the end of the culture period (Z4-megalopa), water is exchanged on a flow-through basis to dilute waste and reduce encounters between larvae and thus the risk of cannibalism.

In order to maintain good water quality, the total water renewal is generally 5-8 times the rearing volume in an intensive rearing system (Yu Xuequan, personnel comm.). Such high water renewal rate tends to make the whole system unstable in terms of water quality parameters (e.g. DO, ammonia and nitrite concentration, pH and temperature, etc.) and microbial community in the water volume. Recently, so called Ecological Larviculture Techniques were attempted in *Eriocheir sinensis* larviculture. The water exchange is minimized in such a system and proper water quality is maintained through the use of biological, chemical and mechanical filtration in a closed recirculation system. Microalgae and rotifer production can also be achieved in this system. Photosynthetic bacteria (PSB) and other probiotic bacteria are usually supplemented to the recirculation system (Wang and Yan, 1999). The larval rearing results in this system tend to be better

and more reliable compared to the batch system due to improved and more stable water quality and the reduction of stress to the crab larvae.

Progressive salinity reduction

In nature, the mitten crab larvae migrate into estuaries as megalopa and move further upstream to rivers and lakes where they grow up to adults. Seawater is thus commonly diluted at the end of the zoea stage or the beginning of the megalopa stage in the hatchery. A two-step dilution is successfully used in practice, with daily salinity reduction of 2 g L^{-1} at Z5, and 3 g L^{-1} at megalopa (Xing et al., 1998). The salinity should be reduced to less than 5 g L^{-1} for 6-7 day-old megalopa. After dilution, the megalopa are usually sold to a "coin-size crab culture farm" or are further reared in the hatchery till juveniles. Megalopa can tolerate long distance transportation (survival up to 90% during 24 h transportation), when placed on a surface of moist water grass or wet towels. The optimum transportation temperature is 14-18 °C.

Larval rearing system

Intensive larval rearing techniques

Intensive larval rearing techniques are mostly performed in inland and coastal hatcheries using different sources of water, such as artificial seawater, underground brackish water, natural seawater and diluted brine. Artificial seawater is applied in inland hatcheries where natural seawater is not available (Zhao, 1980). Although the higher production cost is partially compensated for the proximity of the growout ponds or lakes thus lower transportation costs for the larvae, the complexity and high cost of the saline water formulation still makes this practice uneconomical. Underground brackish water is used in hatcheries near the coast, where this resource is easily available. It has a similar chemical composition as natural seawater, but with a lower bacterial and viral load. However, its ionic composition and salinity need to be adjusted for larval rearing (Zhu et al., 1997; Liu and Gao, 2004). The main water sources used in

Eriocheir sinensis larviculture are natural seawater and diluted brine (60-150 g L^{-1}). Seawater is commonly taken in offshore, then chlorinated for an extended period (overnight) and dechlorinated with sodium thiosulphate. Ozone treatments and recirculation systems are seldom used in local mitten crab hatcheries.

Indoor intensive larval rearing is usually performed in concrete tanks with a typical size of 5m×5m×2m l×w×d. Temperature and aeration are closely controlled in the rearing tanks and sufficient food (e.g. algae, egg yolk, live rotifers and *Artemia*, as well as frozen rotifers, copepods and *Artemia* biomass) is supplied. The stocking density of Z1 varies widely depending on the different larval rearing systems. Lower stocking densities are usually applied in inland hatcheries, while higher stocking densities are preferred in coastal hatcheries where live food and water can be supplied in sufficient quantities. Z1 can be held at high densities (up to 1 million m^{-3}) for a limited period but then they need frequent feeding in order to maintain optimal rotifer density. Moreover, such high densities need to be reduced before the Z5 stage in order to prevent cannibalism. Yields are up to 0.15-0.5 kg megalopa m^{-3} megalopa at stocking densities of 0.2-0.5 million Z1 m^{-3}, and 0.5-0.9 kg at stocking densities of 0.5-1 million Z1 m^{-3}. Survival is usually about 10-15% from Z1 to megalopa.

Although intensive larval rearing techniques are widely practiced in China, the high microbial load, unstable water quality, discharge of waste water and the high production cost urge for more economical and ecological-friendly ways for rearing *Eriocheir sinensis* larvae.

Extensive larval rearing techniques (mesocosm system)

Extensive larval rearing systems are usually managed at low stocking densities in outdoor earthen ponds (often in abandoned shrimp ponds), where the fluctuations of water temperature and salinity are similar to those occurring in the wild. Therefore the quality of the larvae is considered to be similar to wild larvae with better resistance to disease and extreme culture conditions, and thus they have a higher market price. A

comparative study showed the ratio of output and input is 45% higher for outdoor ponds than for indoor tanks (Pang, 1999). Early larval stages (Z1 and Z2) feed on natural food sources available in the ponds, e.g. microalgae, rotifers and other zooplankton; and later larval stages (Z3, Z4, Z5 and megalopa) feed minced fish and *Artemia* biomass (Wu et al., 2006). The size of earthen ponds is between 0.06 to 1 hectare, with a stocking density of 0.02-0.1 million Z1 m^{-3}. The yield of megalopa can be 2-10 kg per hectare depending on the food availability in the ponds (Wu et al., 2006). The less complicated management and the simpler facilities required make this technique widely adopted by local farmers since early 2000. However, the yield in these extensive rearing systems is usually low and unstable. Apart from the fluctuations of water quality parameters, other plankton such as mysids, copepods, *Euphtes* sp., *Didinium* sp., *Vorticella* sp. and *Polydora ligni* larvae may exist in the water column and compete for space, food and DO with crab larvae, thus affecting the metamorphosis and larval survival (Li X.D. et al., 2000; Zeng et al., 2003; Wu et al., 2006).

Green water techniques are usually used in both outdoor earthen ponds and indoor concrete tanks, which is thought to provide an "ecological balanced" or "mature" culture medium. The beneficial impact of micoalgae in a rearing system include the maintenance of the quality of live food, the stabilization of pond water quality via either ammonia uptake and oxygen production and the supply of natural antibiotics (Truong, 2005). In practice, the natural water is disinfected by bleach and lime to kill pathogens and predators, and is allowed to stabilize for several days. Then the desired microalgae need to be inoculated in the culture medium. Except for experimental purposes, clear water systems are seldom applied in practice, and seem to result in small and black larvae (Yu Xuequan, personnel comm.). However, algal blooms may become problematic when using green water, particular in outdoor cultures where they have to be controlled by shading.

Larval nutrition

Like other crustaceans, *Eriocheir sinensis* larvae exhibit an intermittent growth pattern.

Their body weight increases considerably after each molt or metamorphosis (e.g. 120% for Z4 and 200% for megalopa) (Liu et al., 1999). The digestive tract of larvae develops gradually from Z1 to megalopa. The fastest development is observed for Z2 with a tract length increase of 42.05%, during which larvae adapt an exogenous food source when the embryonic reserve is nearly exhausted (Du et al., 1992). The development of the larval digestive system is a physiological adaptation to the feeding habits at each zoeal stage: a simpler elliptical stomach at the early larval stages (Z1 and Z2) is adapted to an omnivorous feeding behavior; while the more differentiated structures developed at later larval stages (Z3 to megalopa) remarkably increases the digestive capacity and fits a more carnivorous feeding behavior. On the other hand, also the enzymatic activities of the digestive enzymes change drastically for each larval stage. Amylase has the strongest enzymatic activity at Z2, whilst activities of trypsin and pepsin reach the highest value at Z3. The overall enzymatic activities of lipase and cellulase are much lower than that of the above mentioned enzymes, and remain constant for the whole larval period (Pan and Wang, 1997).

Early larval stages (particularly Z1) ingest micro-algae when swallowing water. Several genera of microalgae, including *Nannochloropsis*, *Chlorella* sp., *Isochrysis*, diatoms (*Melosira* sp.), *Phaeodactylum tricornutum*, *Chaetoceros*, etc., are used as supplementary food for early larval stages. In addition, algae are also added to the rearing system for the sake of water quality "conditioning" and for maintaining the health of rotifers and *Artemia*. The algal densities applied in the rearing medium range from 0.1 to 2 million cells mL^{-1} depending on their cell size. Although the presence of microalgae in the water improves the survival of Z1, they cannot molt to Z2 solely on it. Starvation results in significantly lower survival and delays larval development. The point of no-return (PNR) for *Eriocheir sinensis* Z1 is 4 days and PNR_{50} is 48 h (Liu et al., 2005). Z1 are not able to molt to Z2 without feeding, but can survive up to 142 h (author's personal observation).

Eriocheir sinensis larvae accept inert feed. Formulated diets modified from larval shrimp diets, dried *Artemia* flakes and decapsulated *Artemia* cysts (Yu et al., 1999) have

been used in *Eriocheir sinensis* larviculture with some success. Meanwhile, egg yolk, soybean milk, frozen rotifers and copepods are also used as supplementary diets. Zhang et al. (2001) reported that the highest megalopa survival was obtained when feeding *Eriocheir sinensis* larvae (from Z2 onwards) formulated diets containing 55.28% protein, 17.39% lipid, 2% vitamins and 6% minerals, in which albumin and white fish meal were used as protein sources, soybean and fish oil (in ratio of 1:3) as fat sources, α-starch as carbohydrate source.

Nevertheless, rotifers and *Artemia* are still the most widely used live foods in the hatcheries. In practice, Z1 and Z2 are usually fed rotifers, while *Artemia* nauplii are usually introduced from Z3 onwards. Ongrown and adult *Artemia* provide a large sized prey item and are fed to Z5 and megalopa. Wu et al. (2007a) suggested that *Centropages dorsispinatus* and *Neomysis japonica* can be used as a more cost-effective live feed to replace *Artemia* nauplius during later larval rearing period (Z4 to megalopa stage).

Food ingestion rate increases step-wise along with larval development. He and Gu (1988) reported the *Artemia* ingestion rate of individual *Eriocheir sinensis* zoea larvae was 11 at Z1, 34 at Z2, 55 at Z3, 91 at Z4, 187 at Z5 and 968 at the megalopa stage in between ecdysis; while a lower daily ingestion rate was obtained in communal rearing system: 1.3 at Z1, 2.9 at Z2, 4.8 at Z3, 8.4 at Z4, 23.5 at Z5 and 49.3 at the megalopa stage (Feng and Zhu, 2000). Yin and Wang (2003) studied rotifer feeding rations with supplementation of microalgae *Cyclotella meneghiniana* and suggested that 20 rotifers mL^{-1} is suitable for Z2 and 30 rotifer mL^{-1} is suitable for Z3 stage. Jiang et al. (2000) suggested that rotifers (40 and 60 ind mL^{-1} for enriched and un-enriched rotifers, respectively) are suitable for Z1 and Z2; and *Artemia* at a density of 10 ind mL^{-1} should be offered from Z3 onwards. Insufficient feeding or overfeeding results in higher mortality and inferior growth (Jiang et al., 2000; Yin and Wang, 2003).

HUFA seems to be important for larval development and survival of mitten crab. Supplementation of algae with high HUFA content such as *Nannochloropsis oculata* results in higher larval survival (Shen and Huang, 1999). Chen et al. (2000) and Jiang et

al. (2000) reported that boosting the dietary n-3 HUFA in rotifers resulted in better growth and survival of *Eriocheir sinensis* zoea larvae. And better resistance to osmotic stress was observed when larvae were fed *Artemia* nauplii containing higher level of n-3 HUFA (Xin et al., 1999).

3. Bottlenecks to the commercial hatchery production

A characteristic of mitten crab larval culture is the highly variable survival, especially when molting from Z1 to Z2, and metamorphosis from Z5 to megalopa. Successful batches in a hatchery can reach an average survival of 10-15%, with maximum of 30-50% survival up to megalopa, but total mortality before Z2 or megalopa stage also frequently occurs. Mitten crab can be considered as an "r" type reproduction strategy species, which spawns and hatches a large number of eggs but these larvae are subjected to high mortality (Davis, 2003). Although massive mortalities could be compensated for maintaining a large broodstock and stocking higher numbers of larvae, this would lead to a substantial waste of time and resources. The variable yield in mitten crab hatcheries may be attributed to different factors:

Differences in batch quality may be a factor contributing to the unstable yield in the hatchery. Mitten crab broodstock are usually fed natural foods during gonad maturation, which may cause nutritional unbalances or malnutrition of the broodstock, and consequently affect the egg and larval quality at hatch. Thus development of standardized optimal broodstock diets is crucial to improve and sustain hatchery production. However, little knowledge on the use of formulated diet or the nutritional requirements of the broodstock is available. It is well known that the lipid class composition and fatty acid profiles of the dietary lipids are crucial, especially in broodstock diets (Harrison, 1990; 1997). Except for a few studies on the effects of lipid and vitamin sources on the reproductive performance of mitten crab broodstock (Wen et al., 2002c; Ai et al., 2003; Wu et al., 2007b), little is known on the effect of dietary HUFA and phospholipids supplementation in broodstock diets on the reproductive performance, egg quality and larval performance of *Eriocheir sinensis*.

Cannibalism often accounts for large mortalities at metamorphosis from Z5 to megalopa, which is mostly caused by asynchronous metamorphosis. The remaining Z5 are usually predated by newly-metamorphosed megalopa. In practice, although cannibalism can be reduced by increasing feeding ration and feeding larger prey such as juvenile or adult *Artemia* at the Z5 and megalopa stages, molting synchrony can only be achieved by providing the smaller larvae with more nutritious feeds and optimizing the feeding regime.

Bacterial interaction is considered to be a critical issue for the commercial development of mitten crab larvae culture. In intensive larval rearing systems, larvae are subject to infections by many opportunistic pathogens including *Pseudomonas putrefaciens, Vibrio* spp., filamentous bacteria, protozoa (e.g. *Zoothamnium* sp.), through attachment on the body surface and epithelia of the digestive organ or directly entering via wounds (Wu and Feng, 2004). Death may be caused by hypoxia or by interference with molting, locomotion or feeding. Under stressful conditions, such as poor water quality, unfavorable rearing conditions and feeding, and nutritional deficiency, the level of those pathogens rise rapidly and become deleterious to mitten crab larvae, particularly to the early larval stages. Although a certain survival rate can sometimes be recovered by reducing larval density and increasing water renewal, the mortality is usually high once the larvae get infected.

Antibiotics have been used to treat infected crab larvae in earlier years. But the use of these chemicals is nowadays strictly regulated in aquaculture due to a number of negative side effects in aquatic animal health (i.e. development of multiple antibiotic resistance and increased virulence). Though it is not as effective as antibiotics, the use of formalin can also promote survival of the larvae as it facilitates molting, and acts as therapeutic and prophylactic for diseases caused by protozoa and bacteria (author's unpublished data). Probiotics and immunostimulants are also potentially useful tools in the disease prevention and control, although their effectiveness is sometimes questioned.

The shortage of broodstock and juveniles of good quality and the high market price of mitten crab has triggered the frequent exchange of resources among the different geographical populations (mainly transport from the Liaohe and Ou River area to the Yangtze River basin) in the 1990s, which consequently has reduced the genetic diversity of this species due to the high level of gene flow (Qiu, 1998; Wang and Li, 2002). Although these large scale exchanges are nowadays seldom carried anymore, the care for genetic diversity is crucial for conservation of this resource.

Chapter III

Population genetics

Chapter III

Genetic diversity and population structure of Chinese mitten crab *Eriocheir sinensis* in China

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Abstract

The Chinese mitten crab *Eriocheir sinensis* is an indigenous and economically important species in China, yet it can also be found as invasive species in continental Europe and America. Mitten crab aquaculture has been exploited extensively as food resources since the 1990s. Despite the ecological and economic importance of the species, the genetic structure of the native mitten crab population is not yet well understood. In this paper, we investigated the genetic composition of mitten crab populations in mainland China by screening samples from nine locations covering six river systems at six microsatellite loci. As an additional step, we also integrated the current data with those reported by Herborg et al. (2007) (*Molecular Ecology* 16, 231-242) on three representative mitten crab populations from Europe and America, and one native sample from Liaohe.

Less structuring among the native populations indicated that high gene flow occurred between geographically remote river systems, which is considered as a consequence of human relocation (e.g. transport and escapees from hatchery, culture ponds and net enclosures). Conservation polices should emphasize the protection of localized populations and cessation of human-facilitated introduction. On the other hand, it further confirmed that a founder effect and a stepping stone colonization from China to Europe to North America were obvious in the invasive populations as observed by a gradually decrease in expected heterozygosity (H_E) and a reduced allelic richness (A), compared to the native populations.

Keywords *Eriocheir sinensis*; genetic diversity; microsatellite DNA; population genetics

1. Introduction

The Chinese mitten crab, *Eriocheir sinensis* is a euryhaline brachyuran with a catadromous life cycle, consisting of an oceanic planktonic zoea larval stage, an

estuarine megalopa stage, a freshwater juvenile-adult stage, and a return to the marine environment as an adult to mature and spawn. Its native range extends from the eastern pacific coast of China, 24 ºN northwards to the Korean Peninsula, 42-43 ºN; from 124 ºE westwards to 112 ºE (Gu and Zhao, 2001). Nevertheless, in past 100 years, it has been introduced inadvertently into continental Europe, United Kingdom and San Francisco Bay via shipment and has become one of the most notorious aquatic invaders in these areas (Carlton, 1985; Cohen and Carlton, 1997; Herborg et al., 2003; Rudnick et al., 2003).

The mitten crab is an economically important freshwater crustacean species in China. Exploitation of its natural populations has a long tradition. In earlier years, the megalopa were usually captured from river estuaries during their upstream migration and restocked in the open lakes for grow-out (Zhang et al., 2002; Liu et al., 2007). However, owing to the intensive exploitation and irrigation works, natural stocks have decreased dramatically since the 1960s. The natural yield of megalopa and fishery landings of adult mitten crabs in the Yangtze River estuary dropped to less than 10 kg and 15 mt respectively in the mid of the 1980s (Yu et al., 1999). Nevertheless, benefiting from advances in seed production and larviculture techniques (Zhao, 1980), mitten crab aquaculture in China has developed extensively since the early 1990s, with annual aquaculture production of 500,000 mt in 2005 (FAO, 2007). Nowadays, few wild caught megalopa or juveniles are used in the farm. Instead, those produced from hatcheries are the major source for mitten crab farming, which are usually reared in outdoor earthen ponds or released into net enclosures in lakes for grow-out.

In its native range, the mitten crab is mainly distributed in three main drainages, the rivers Liaohe, Yangtze and Ou and a number of smaller adjacent river systems (Zhang et al., 2000). Stocks originating from the Yangtze River basin are commercially the most important resource due to a number of attributes including better growth patterns, larger size and good flavor (Li Y.S. et al., 2000). However, there has been a shortage of broodstock and juveniles from the Yangtze River basin due to over-exploitation, which has in the past driven the frequent stock transfer among the different geographical

populations (mainly transport from Liaohe and Ou River area to Yangtze River basin) in the 1990s. Further to such deliberate stocking attempts it is likely that escapees of unknown (genetic) origin from hatcheries, culture ponds or net enclosures in lakes have occurred. Thus anthropogenic dispersal and restocking programs may have caused a high level of gene flow and a potential threat for the genetic diversity of the native populations (Qiu, 1998; Wang and Li, 2002).

The genetic characterization of stocks has long been a major concern in fisheries resources management (Valles-Jimenez et al., 2005). Molecular markers have provided fisheries managers with a tool that unequivocally identifies species and determines spatial genetic structure of stocks providing crucial information to design and manage sustainable fishing practices (Ward, 2000). A number of studies on the native mitten crab populations described some differences among the geographically distinct populations using enzymatic proteins and molecular markers (Wang and Yu, 1995; Gao and Zhou, 1998; Zhou and Gao, 1999). However, a conclusive pattern of geographical population structure has not yet emerged. The limitation of the methodologies applied in previous studies, such as low polymorphism at the analyzed allozyme loci and low repeatability for RAPD markers, may limit their value for detecting population differentiation. Such problems can potentially be avoided by analyzing hyper-variable microsatellites loci which can be used to detect low levels of population structure. Microsatellite markers have been successfully employed in marine species with high levels of gene flow such as whelks and cod (Hutchinson et al., 2001; Weetman et al., 2006). They have been further proved to be useful tools to investigate the population divergence and colonization process of *Eriocheir sinensis* in its invasive range (Herborg et al., 2007).

The aim of this study was to use microsatellite markers to describe the genetic differentiation, dispersal patterns and gene flow among the native mitten crab populations; and to provide better knowledge of genetic diversity and structure of this over-exploited species. These data could provide vital input in support of sustainable exploitation and conservation of the natural populations.

2. Materials and Methods

2.1 Sample collection and geographical information

17 to 55 *Eriocheir sinensis* specimens, fished directly from the rivers, were collected between 2001 and 2002, from nine locations in mainland China. They are geographically distributed in six river systems, namely River Liaohe (PJ), Haihe (HH), Huanghe (KL), Yangtze (RD, YZ, NJ and YR), Ou (WZ) and Nanliujiang (HP) (Table 1). The Yangtze River is at the center of the distribution. The River Liaohe, Haihe and Huanghe are northern locations. The River Ou and Nanliujiang are southern locations (Fig.1). In addition, data set from three representative invasive *Eriocheir sinensis* populations sampled from River Elbe, Germany (Elbe), River Thames, UK (Thames) and San Francisco Bay, USA (SF), and a native population sampled from the River Liaohe (Liahoe) (Herborg et al., 2007), were also integrated in the result and discussion in this chapter for macro-geographical comparison and bias introduction control by combining two data sets.

2.2 Microsatellite amplification and screening

The pleopod of the adults or juvenile crabs were taken and preserved in 98% ethanol for subsequent DNA extraction. Total genomic DNA was extracted using a modified cethyltrimethyl ammonium bromide (CTAB) protocol based on Doyle and Doyle (1985) (Table 2). The samples were genotyped at six dinucleotide microsatellite loci *Esin42*, *Esin55*, *Esin67*, *Esin74*, *Esin75* and *Esin87* as described in Hänfling and Weetman (2003). Polymerase chain reaction (PCR) was carried out in 10 μL and contained 10-50 ng DNA each. The same PCR protocols and reaction conditions as described in Hänfling and Weetman (2003) were used for each locus. PCR products were run on Pharmacia ALF Express automated sequencers (Amersham, UK) with at least three internal size standards included to permit reliable size-scoring using FRAGMENT MANAGER 1.2 (Amersham, UK). Additionally, two previously genotyped individuals from the data set of Herborg et al. (2007) were included on each gel to improve cross-referencing among gels and data sets. Raw data were checked for scoring errors due to stuttering or larger allele dropout using the program MICRO-CHECKER 2.2.3

(Van Oosterhout et al., 2004).

Table 1. Information of the native and invasive *Eriocheir sinensis* populations sampled for current study

	Location	Number of individuals	Geographical information		
			Latitude	Longitude	River system
Native *E.sinensis* populations	Panjin (PJ)	27	41°00'N	122°07'E	River Liaohe, China
	Liahoe	49	41°21'N	122°38'E	River Liaohe, China
	Haihe (HH)	55	39°13'N	117°20'E	River Haihe, China
	Kenli (KL)	30	37°59'N	118°54'E	River Huanghe, China
	Rudong (RD)	45	32°30'N	121°80'E	River Yangtze, China
	Yizheng (YZ)	30	32°27'N	119°16'E	River Yangtze, China
	Nanjing (NJ)	30	32°04'N	118°78'E	River Yangtze, China
	Shanghai (YR)	44	31°10'N	121°28'E	River Yangtze, China
	Wenzhou (WZ)	18	28°02'N	120°65'E	River Ou, China
	Hepu (HP)	19	21°33'N	109°20'E	River Nanliujiang, China
Invasive *E.sinensis* populations	Elbe	85	52°45'N	12°02'E	River Elbe, Germany
	Thames	47	51°27'N	0°25'E	River Thames, UK
	SF	16	37°39'N	122°22'W	San Francisco Bay, USA

Table 2. Allele properties of the six microsatellite loci used in current study (as described in Hänfling and Weetman, 2003)

Locus	Gene bank accession No.	Repeat motif	Ta [cycles]
Esin 42	AF536345	$(GT)_{19}$	55[35]
Esin 55	AF536346	$(GT)_4AT(GT)_2GA(GT)_8AT(GT)_2GAT(GT)_{18}$	55[40]
Esin 67	AF536347	$(GT)_{11}$	58[35]
Esin 74	AF536348	$(GT)_{19}(GT)_{19}$	55[40]
Esin 75	AF536349	$(AC)_{10}TC(AC)_3$	52[35]
Esin 87	AF536350	$(GT)_4GGTG(GT)_{13}$	55[40]

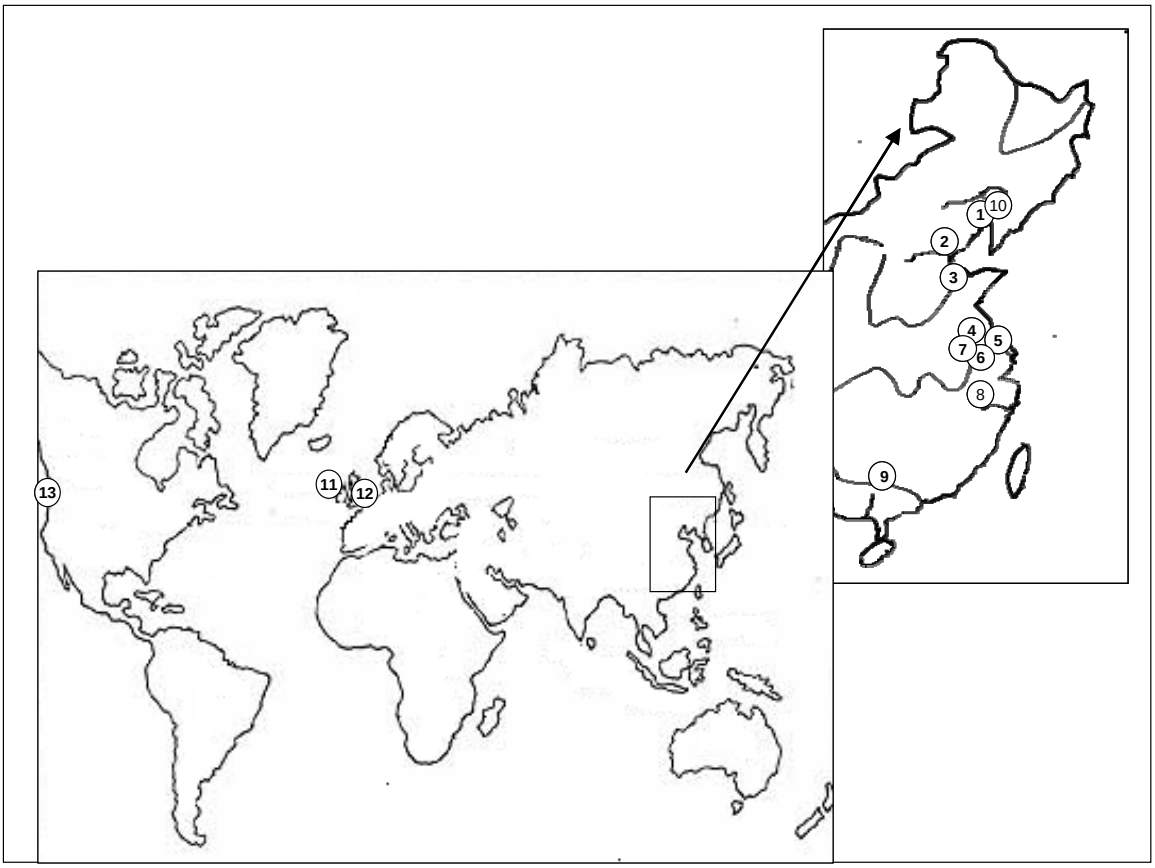

Fig. 1. Map of the native and invasive *Eriocheir sinensis* populations sampled for current study
River Liaohe: (1) PJ; (10) Liahoe;
River Haihe: (2) HH;
River Huanghe: (3) KL;
River Yangtze: (4) RD; (5) YZ; (6) NJ; (7) YR;
River Ou: (8) WZ;
River Nanliujiang: (9) HP;
River Thames: (11) Thames;
River Elbe: (12) Elbe;
San Francisco Bay: (13) SF

2.3 Data analysis

The observed and unbiased expected heterozygosities (H_O and H_E; Nei, 1978) were estimated using GENEPOP 3.2 (Raymond and Rousset, 1995) and allelic richness (A) was estimated by rarefaction, as the expected average number of alleles at the size of the smallest using FSTAT 2.9 (Goudet, 1995). Deviations from Hardy-Weinberg equilibrium were tested via separate one-tailed test for heterozygote excess and deficiency for each locus in each population, to obtain unbiased estimates of the exact

p-value in GENEPOP 3.2 with 100 batches of 5000 iterations. Linkage disequilibrium in the form of independence of loci within populations was tested using the Markov chain method (3000 iterations) and Fisher's exact test (Haldane, 1954).

Genic differentiation for each population pair across all loci was estimated using GENEPOP 3.2 by comparing allele frequencies between samples using Fisher's exact test. θF_{ST} (Weir and Cockerham, 1984) estimators of *F*-statistics were calculated using GENEPOP 3.2 in order to quantify the distribution of genetic differentiation between pairs of populations and as a global estimate.

Principal Component Analysis (PCA) was used to produce a visual representation of differentiation among the global populations and the native populations, using the program PCA-GEN (available from J. Goudet at http://www2.unil.ch/popgen/softwares/pcagen/htm).

Isolation by distance analysis was carried out by comparing matrices of genetic population divergence with geographical distance, which was then transformed to $F_{ST}/1\text{-}F_{ST.}$ The geographical distances were calculated by the shortest sea distance between each pair of samples based on their localities.

The program STRUCTURE 2.1 (Pritchard et al., 2000) was used to determine the number of genetically different groups amongst the Chinese samples. This approach is an individual based Bayesian cluster analysis which does not consider *a priori* defined populations and can therefore potentially detect population structure which is obscured through secondary admixture. STRUCTURE estimates the posterior probability *P*r (X/K) that the samples were drawn from K populations. The program was run 3 times independently for a range of K between 1 and 8, with 500,000 MCMC repetitions and a 'burn-in' of 10,000 iterations using both the admixture and non-admixture model of population structure. In all cases the correlated allele frequency model was used.

3. Results

MICRO CHECKER found no evidence of scoring errors in any sample. And no evidence of linkage disequilibrium was observed at any of the six loci investigated, thus allowing allelic variation at all loci to be treated as independent.

The six microsatellite loci showed varying numbers of alleles, ranging from 18 alleles in locus *Esin87* to 47 alleles in locus *Esin55* (Table 3). Mean H_E and H_O across loci for the native populations varied respectively from 0.77 (HP) to 0.85 (NJ), and from 0.74 (YZ) to 0.85 (NJ), which were comparable to the European populations (0.80 and 0.78 for Elbe; 0.79 and 0.73 for Thames, respectively); whilst relatively low H_E and H_O (0.66 and 0.68, respectively) were observed for the American population (SF). The mean allelic richness for the native populations ranged from 9.36 (HP) to 13.19 (NJ), which were higher than for the Elbe (7.87), Thames (7.50) and SF (5.17) populations. The mean $H_{E,}$ H_O and allelic richness across loci for the native Liahoe population obtained from the previous study (Herborg et al., 2007) (0.82, 0.76 and 12.89, respectively) were in the range of the native populations genotyped in the current study, which indicated that no bias was introduced by combining two data sets in this study.

The global F_{ST} values were 0.037 among the worldwide distributed mitten crab populations and 0.0073 among the native populations, indicating stronger genetic differentiation among the invasive and native populations than among the native populations.

Table 3. Genetic diversity of the native and invasive *Eriocheir sinensis* populations at 6 microsatellite loci

Name of populations	Genetic diversity							
		Esin42	*Esin55*	*Esin67*	*Esin74*	*Esin75*	*Esin87*	Mean
Panjin (PJ)	H_E	0.91	0.93	0.72	0.93	0.96	0.50	0.82
	H_O	0.81	0.96	0.70	0.96	0.93	0.44	0.80
	A	10.65	16.22	7.63	15.45	18.89	6.48	12.55
Haihe (HH)	H_E	0.89	0.95	0.81	0.93	0.96	0.46	0.83
	H_O	0.85	0.92	0.81	0.94	0.94	0.45	0.82
	A	12.13	17.11	7.44	15.00	19.19	5.78	12.78
Kenli (KL)	H_E	0.91	0.95	0.71	0.95	0.96	0.55	0.84
	H_O	0.87	0.97	0.77	0.93	0.90	0.57	0.82
	A	12.22	17.10	7.94	17.14	18.29	5.04	12.96
Rudong (RD)	H_E	0.90	0.95	0.68	0. 90	0.96	0.37	0.79
	H_O	0.82	0.93	0.67	0.93	0.96	0.38	0.78
	A	11.09	17.87	6.42	14.07	18.29	4.89	12.11
Yizheng (YZ)	H_E	0.91	0.96	0.75	0.91	0.96	0.36	0.81
	H_O	0.83	0.96	0.73	0.87	0.76	0.33	0.74
	A	11.78	19.13	10.05	15.02	17.85	5.25	13.18
Nanjing (NJ)	H_E	0.91	0.94	0.82	0.94	0.96	0.51	0.85
	H_O	0.93	0.93	0.90	0.97	0.87	0.47	0.85
	A	11.88	17.20	8.21	16.00	18.21	7.66	13.19
Shanghai (YR)	H_E	0.90	0.96	0.74	0.96	0.95	0.53	0.84
	H_O	0.82	0.93	0.66	0.93	0.94	0.38	0.78
	A	11.27	18.56	6.36	17.85	17.19	6.84	13.01
Wenzhou (WZ)	H_E	0.90	0.92	0.76	0.93	0.94	0.31	0.79
	H_O	0.89	1.00	0.83	0.83	0.88	0.28	0.79
	A	11.54	16.00	7.75	14.19	15.00	6.33	11.80
Hepu (HP)	H_E	0.87	0.85	0.75	0.92	0.84	0.36	0.77
	H_O	0.95	0.84	0.89	0.95	0.83	0.37	0.81
	A	8.81	9.36	12.38	14.67	7.97	2.98	9.36
Elbe	H_E	*0.85*	*0.88*	*0.81*	*0.84*	*0.80*	*0.60*	*0.80*
	H_O	*0.86*	*0.81*	*0.74*	*0.80*	*0.87*	*0.61*	*0.78*
	A	*9.14*	*9.62*	*6.42*	*8.81*	*8.74*	*4.47*	*7.87*
Thames	H_E	*0.82*	*0.87*	*0.81*	*0.82*	*0.76*	*0.68*	*0.79*
	H_O	*0.84*	*0.70*	*0.67*	*0.80*	*0.75*	*0.61*	*0.73*
	A	*7.77*	*9.85*	*6.47*	*8.26*	*7.63*	*5.02*	*7.50*
San Francisco Bay (SF)	H_E	*0.77*	*0.64*	*0.66*	*0.67*	*0.83*	*0.38*	*0.66*
	H_O	*0.81*	*0.81*	*0.56*	*0.75*	*0.69*	*0.44*	*0.68*
	A	*6.00*	*7.00*	*3.00*	*5.00*	*6.00*	*4.00*	*5.17*
Liahoe	H_E	*0.92*	*0.95*	*0.77*	*0.92*	*0.96*	*0.42*	*0.82*
	H_O	*0.83*	*0.84*	*0.76*	*0.90*	*0.77*	*0.45*	*0.76*
	A	*11.99*	*18.18*	*8.36*	*15.36*	*18.05*	*5.36*	*12.89*
	No.of alleles	21	47	20	34	45	18	
	Allele range (base pair)	223-273	81-179	137-179	137-199	156-258	123-161	

H_E, expected heterozygosity; H_O, observed heterozygosity; *A*, allelic richness; Data in italic are obtained from Herborg et al. (2007)

Table 4. Matrix genetic differentiation using pairwise θF_{ST} among the native and invasive *Eriocheir sinensis* populations and Fisher's exact test of genic differentiation presented as **P*<0.05, ** *P* <0.01, * *P* <0.001**

	PJ	Liahoe	HH	KL	RD	YZ	NJ	YR	WZ	HP	Elbe	Thames	SF
Panjin (PJ)	0												
Liahoe	0.0088 ***												
Haihe (HH)	0.0020 ***	0.0044 **											
Kenli (KL)	0.0018	0.0066 **	0.0053 *										
Rudong (RD)	0	0.0136 ***	0.0063 *	0.0083 **									
Yizheng (YZ)	0.0042 *	0.0147 ***	0.0084 **	0.0060	0.0031 *								
Nanjing (NJ)	0.0048 *	0.0029 **	0.0001	0.0010	0.0098 ***	0.0062 *							
Shanghai (YR)	0.0020 *	0.0073 ***	0.0036 **	0.0001	0.0092 ***	0.0053 *	0.0016 *						
Wenzhou (WZ)	0.0047	0.0073 ***	0.0091 **	0.0085 **	0.0122 ***	0.0047	0.0065 *	0.0088 ***					
Hepu (HP)	0.0324 ***	0.0317 ***	0.0325 ***	0.0339 ***	0.0400 ***	0.0244 ***	0.0244 ***	0.0347 ***	0.0293 ***				
Elbe	0.0553 ***	0.0514 ***	0.0498 ***	0.0460 ***	0.0650 ***	0.0719 ***	0.0447 ***	0.0474 ***	0.0604 ***	0.0782 ***			
Thames	0.0644 ***	0.0637 ***	0.0602 ***	0.0546 ***	0.0794 ***	0.0825 ***	0.0518 ***	0.0541 ***	0.0727 ***	0.0916 ***	0.0098 ***		
SF	0.1091 ***	0.0964 ***	0.1026 ***	0.1033 ***	0.1198 ***	0.1201 ***	0.1003 ***	0.1153 ***	0.1055	0.1100	0.0901	0.1046	

Analysis of genetic variation among samples using pairwise $F_{ST,}$ showed varying levels of differentiation (Table 4). Relatively high pairwise F_{ST} values were obtained between HP population (0.02 to 0.04) and the remaining populations, and between the two European populations (>0.04), American population (>0.1) and the others. Furthermore pairwise Exact Tests of genic differentiation among worldwide populations indicated that the invasive populations and HP were significantly divergent from the other native populations. On the other hand, although lower pairwise F_{ST} levels (<0.01) were observed across most of the native populations, a more significant differentiation among many Yangtze samples (NJ, YZ, YR and RD) and among the Liaohe samples (PJ and Liahoe) was found, compared to the divergence among different rivers.

The grouping of the native and invasive populations in the PCA plot placed most of the native populations relatively close to each other. The invasive populations showed a clearly distinct position from the native cluster. Among the invasive populations the European samples from the Rivers Elbe and Thames cluster closely together (Fig. 2-1). On the other hand, although sampling from the same river system, the samples from different locations along the River Yangtze do not cluster strongly together; and the samples from River Liaohe showed similar separation. Overall the clustering of the native samples seems to show no clear geographical pattern, with exception of the distinct position of the HP samples from the most southern part of the distribution (Fig. 2-2).

Pairwise matrices of $F_{ST}/(1-F_{ST})$ were significantly correlated with geographical distances for the global data set (r=0.8371, *P*r=0.0225<0.05) (Fig. 3-1). However, no significant correlation was detected for the native populations (r=0.5207, *P*r=0.0650>0.05) (Fig. 3-2), suggesting a lack of isolation by distance among samples.

Individual based cluster analysis of Chinese samples using both the admixture and non admixture model showed no consistent difference was found between different values for K across replicate runs and therefore no evidence for hidden population structure (data not shown).

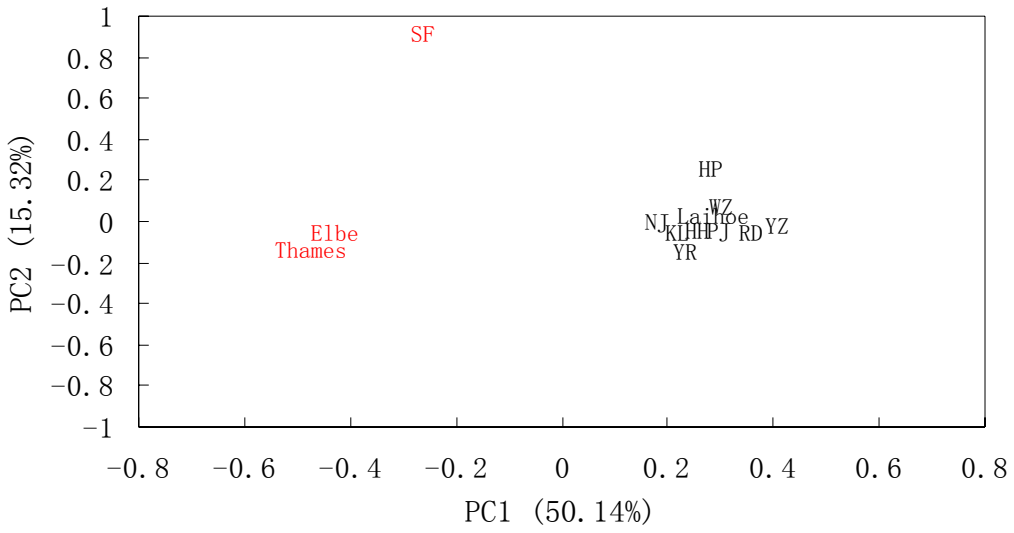

Fig. 2-1. Principle Component Analysis based on pairwise F_{ST} of the native and invasive *Eriocheir sinensis* populations

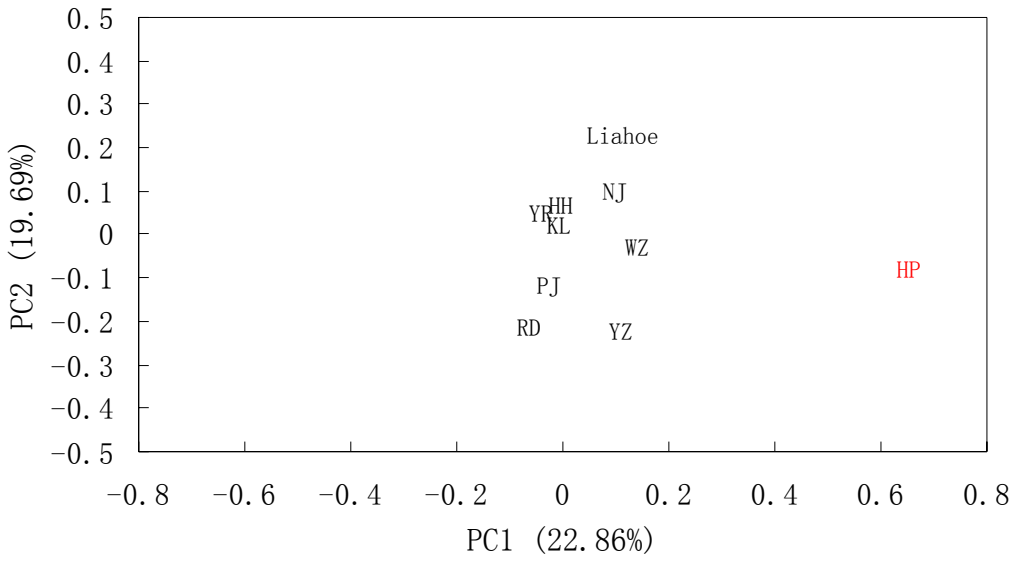

Fig. 2-2. Principle Component Analysis based on pairwise F_{ST} of the native *Eriocheir sinensis* populations

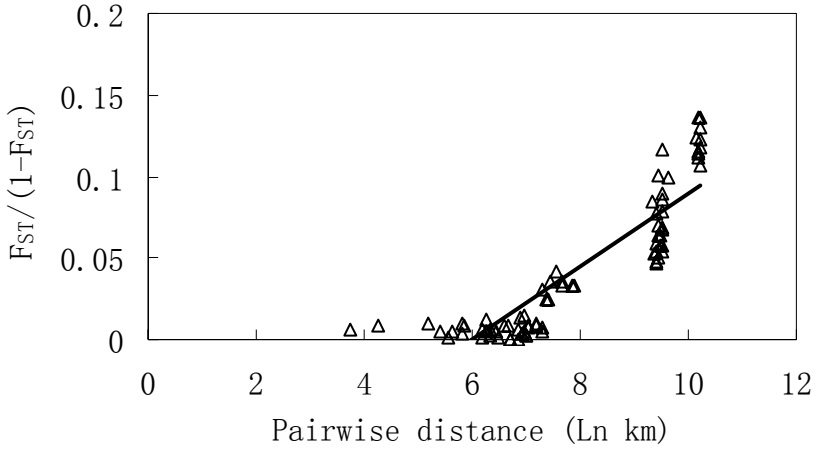

Fig. 3-1. Plot of linearized F_{ST} and pairwise distance among the native and invasive *Eriocheir sinensis* populations (r=0.8871, *P*r =0.0225)

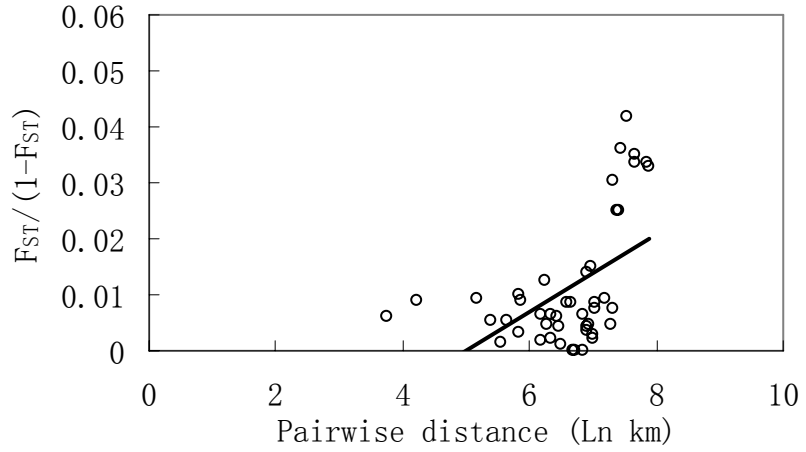

Fig. 3-2. Plot of linearized F_{ST} and pairwise distance among the native *Eriocheir sinensis* populations (r=0.5207, *P*r =0.0650)

4. Discussion

The current study revealed that the HP population sampled from the River Nanliujiang is substantially more divergent to the other native populations. This is in agreement with earlier studies showing that this population deserves species or subspecies status. The mitten crab population from the River Nanliujiang was first recognized as a subspecies

of *E. japonicus* and named as *E. japonicus hepuensis* by Dai (1988; 1991). It was further denominated as *E. hepuensis* by Guo et al. (1997). However, the taxonomy of genus *Eriocheir* has not yet been consolidated due to the inconsistent study results from different groups using different analyzing methods. Li et al. (1993) suggested that both *E. sinensis* and *E. hepuensis* are the same species of *E. japonicus*. Afterwards Li and Zou (1999), Zhao and Li (1999) and Zhou and Gao (1999) reported that *E. hepuensis* belongs to *E. japonicus*, both being different from *E. sinensis*. Whereas more recently, through analysis of the complete sequences of the nuclear DNA internal transcribed spacer (ITS) and portions of the mitochondrial genome corresponding to the COI, Tang et al. (2003) pointed out that *E. hepuensis*, *E. japonicus* and *E. sinensis* are three subspecies of *E. japonica*: *E. j. japonica*, *E. j. sinensis* and *E. j. hepuensis*. Our results agree partially with Tang et al. (2003), indicating that the HP population might belong to a different subspecies with *E. sinensis*.

A number of results indicate that there is no significant geographical population structure among the northern group populations (excluding the HP population from the River Nanliujiang). Firstly, the rather low global F_{ST} value (0.0073) among the native populations compared that among global populations (0.0369), indicates a weak overall structure. Secondly, differentiation among river systems was not significantly larger than differentiation within river systems. Thirdly, population genetic differentiation does not appear to be related to geographical distances since no significant isolation-by-distance relationship was detected among the native populations (r=0.5207, *Pr*=0.0650). Such a pattern indicates a high level of gene flow among the different river systems, not only at a geographical mesoscale (ten to hundreds of kilometers), but also at macroscale (more than thousand kilometers). This result is in fact not unexpected. Driven by the high market price of mitten crab in the Yangtze River area, the transport of broodstock and juvenile from north and south to the hatcheries and farms in this area, and *vice versa*, have been carried out since the early 1990s. Although scientific data about escapees in mitten crab aquaculture are not yet available, the accidental introduction and escape events are known to happen, either due to culture system failure, accidents or carelessness. Therefore the potential threat to the genetic integrity of wild

populations is more relevant when hatchery juveniles obtained from non-local broodstocks are involved. The frequent translocation and escapees could result in high level of gene flow among the geographically distinct populations and reducing the localized genetic diversity.

The current study showed, however, there is relatively large level of differentiation among samples (RD, YZ, NJ and YR) from the River Yangtze and the samples from the River Liaohe (PJ and Liahoe), respectively, compared to the differentiation among river systems. This is unexpected assuming that all samples obtained from the same river system should be a panmictic population (originating from the same spawning population given that spawning takes place in the estuary). It should be stressed that both river systems are the area where mitten crab aquaculture are extensively performed in China. Such intra-specific differentiation may be caused by a recent introduction of megalopa and juvenile escapees from hatcheries and farms. In practice, some farms buy their megalopa and juveniles from wholesale suppliers while other farmers produce by their own using broodstock. As a result, the history and the origin of breeding stocks are most of the time not suitably controlled.

Population genetic structure is clear evident on a more extended geographical scale (among the continents), which confirms the observation by Herborg et al. (2007). A number of results are consistent with the hypothesis that this differentiation has been generated through a stepping stone colonization from China to Europe to North America: i) the significant isolation-by-distance result on a global geographical scale; ii) the close clustering of European and American populations in the PCA plot and iii) European populations exhibited lower genetic diversity than the native populations, whilst the American population displayed the lowest diversity. Furthermore these results are also supported by a previous study based on mitochondrial DNA sequencing (Hänfling et al., 2002). The reduction of genotypic diversity between the native sources and the invasive populations is possibly due to a founder effect for the invasive genotype (Herborg et al., 2007).

In summary, our results show that *E. sinensis* exhibits widespread population structure, but high differentiation only across very large geographical scales (among continents). No sub-structuring populations are identified within the native populations, except for the HP population. The locally commercial exploitation in *E. sinensis* results in less genetic differentiation among the native populations. Thus it is recommended that the translocation of broodstock and juveniles, and any accidental introduction of hatchery samples in the wild represent a potential threat to the genetic integrity of natural populations. As a consequence, cease of human translocation and tracking the broodstock history or genetic background are essential for a sustainable and responsible use of mitten crab resources.

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Chapter IV

Broodstock nutrition

Chapter IV Section I

Effect of dietary soybean lecithin on reproductive performance of Chinese mitten crab *Eriocheir sinensis* broodstock

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Abstract

The effect of increasing levels of dietary soybean lecithin on the ovary development and reproductive performance of Chinese mitten crab *Eriocheir sinensis* were investigated using four semi-purified formulated diets supplemented with 0% (Diet 1), 1.2% (Diet 2), 2.4% (Diet 3) and 3.6% (Diet 4) soybean lecithin. Four groups of 40 females, with an average individual body weight of 95-120 g were fed the experimental diets for a period of 7 months. Male crabs were introduced into the female rearing system in March, and mating, spawning and egg hatching occurred in the following months.

After 10 weeks feeding, the females fed Diet 3 had a significantly higher gonad somatic index (GSI) than the females fed Diet 1, while the females fed Diet 2 had a significantly higher hepatic moisture content and lower hepatic lipid content than the other groups ($P<0.05$). Mating occurred after about 6 month feeding. The spawning rate was higher among the females fed Diet 4 and Diet 3 (95% and 92%, respectively), compared to the females fed Diet 2 and Diet 1 (both 81%). The results showed that egg production (total number of eggs female^{-1}) and fecundity increased with increasing dietary soybean lecithin level, with females fed Diet 1 and Diet 2 having significantly lower values than the females fed Diet 4 ($P<0.05$). Our results suggest that dietary soybean lecithin supplementation has a positive effect on ovarian development and reproductive performance of *E. sinensis* broodstock. Further study should aim to investigate the optimal phospholipids level in the broodstock diet in respect to offspring quality.

Keywords *Eriocheir sinensis* broodstock; phospholipids; soybean lecithin; maturation; fecundity; egg quality

1. Introduction

Chinese mitten crab *Eriocheir sinensis* is an important aquaculture species in China, with an annual production reaching 500,000 tons, valued at 2.2 billion US$ in 2005 (FAO, 2007). Since the 1980's, the natural population of mitten crab in China has

declined sharply due to overfishing, water pollution and migration obstruction from the construction of dams and flood gates between rivers and lakes. Since then, mitten crab has been widely cultured and stocked in ponds, reservoirs and lakes. Although significant advances have been made in larval rearing and growout of *E. sinensis* (Zhao, 1980), the variable quality of eggs spawned by captive broodstock remains a considerable constraint for the hatchery production of larvae and postlarvae in many commercial hatcheries.

The nutritional status of crustacean broodstock greatly influences gonad maturation, reproductive performance, and egg and offspring quality (Harrison, 1990; Lavens and Sorgeloos, 2000; Wouters et al., 2001a). Studies on the nutritional requirements of broodstock could therefore lead to improved reproductive output and larval quality. Current practice in commercial mitten crab hatcheries is to feed broodstock a diet of fresh food such as trash fish, clam and sandworm. The nutritional value of this diet is highly variable and it furthermore decays rapidly and deteriorates water quality, and hence increases the risk of disease introduction. Although recent research has shown that it is possible to completely substitute fresh food with formulated diets for *E. sinensis* broodstock (Wen et al., 2002c; Wu et al., 2004; Wu et al., 2007b), such development is hindered by the lack of knowledge of the nutritional requirements of broodstock which precludes formulation of a diet of optimal composition.

Phospholipids (PL) are considered a essential nutrient in crustacean diets, as they are associated with the molecular organization of cells, especially membranes, and play a vital role in the transfer of lipids from the hepatopancreas to the ovary via hemolymph during ovary development (Harrison, 1990). However crustaceans only have a limited ability to biosynthesize PL *de novo* (Shieh, 1969; Teshima and Kanazawa, 1979; Kanazawa et al., 1985), and supplementation of dietary PL therefore appears to be crucial for cultured crustacean species. Although many studies have been conducted on the PL requirements of larval and juvenile crustaceans, such as lobster *Homarus americanus* (Conklin et al., 1980), penaeid shrimp (Chen, 1993; Coutteau et al., 1996; Paibulkichakul et al., 1998; Thongrod and Boonyaratpulin, 1998), freshwater prawn

Macrobrachium rosenbergii (Briggs et al., 1988) and mitten crab *E. sinensis* (Cheng et al., 1998c; Wang et al., 2004), only limited studies have investigated the dietary PL requirement of crustacean broodstock. Millamena et al. (1986) were the first to demonstrate that fecundity and survival of *Penaeus monodon* broodstock were positively affected by the inclusion of soybean lecithin into the diets. Similar results were found in *Litopenaeus stylirostris* broodstock when the diet was supplemented with 1.5% soybean lecithin (Bray et al., 1990). Furthermore, higher hatching rates, larval production and spawning frequency were obtained when increasing levels of PL were added to the diets of *Litopenaeus vannamei* broodstock (Cahu et al., 1994), which suggested that 2% PL should be supplemented in the broodstock diets in order to maintain high spawning frequency and fecundity of *L. vannamei*. The present study investigated the effect of dietary PL levels on reproductive performance of *E. sinensis* broodstock in terms of maturation, fecundity and egg quality.

2. Materials and Methods

2.1 Experimental diets

Four semi-purified diets were formulated to contain different levels of soybean lecithin. The constant and variable ingredients of the experimental diets are shown in Table 1. Squid meal and soybean protein isolate were defatted with ethanol to remove traces of lipid prior to preparing the experimental diets. In order to maintain the same total lipid level and fatty acid composition in all diets, varying levels of soy lecithin were replaced by soybean oil in the different diets. Dry ingredients were thoroughly mixed and lipid ingredients were added drop by drop to the dry mixture while blending. All ingredients were mixed with 20% (w/w) hot water. The diets were extruded through a 4-mm orifice die and dried with the use of electric fan at room temperature for 48 h. All experimental diets were then stored at -20 °C until use. Upon feeding, the feeds were broken into 1.5-2 cm pieces to facilitate the grasping by the crabs.

Table 1. Formulation of the experimental diets supplemented with different levels of soybean lecithin

Constant ingredients	%			
Casein	30.0			
Crude corn starch	26.1			
Gelatin	10.0			
Defatted squid meal powder	7.0			
Defatted soybean protein isolate	9.4			
Protein extract	2.0			
Fish oil [a]	2.5			
Cholesterol	0.5			
Sodium alginate	2.0			
Vitamin mix [b]	1.0			
Mineral mix [c]	3.0			
Choline chloride	1.0			
Inositol	0.6			
Ascorbic acid phosphate ester [d]	0.3			
Tocopherol acetate [e]	0.1			
Butylated hydroxytoluene (BHT)	0.01			
Variable ingredients	Diet 1 (0%)	Diet 2 (1.2%)	Diet 3 (2.4%)	Diet 4 (3.6%)
Soybean lecithin [f]	0	1.2	2.4	3.6
Soybean oil	4.5	3.3	2.1	0.9

[a] Fish oil: contained 60% HUFA, DHA/EPA = 1; Chemport Co., Ltd, Korea

[b] Vitamin mix: 1 kg of diet contained vitamin A 10, 000 IU; vitamin D 2, 500 IU; vitamin K 64 mg; thiamin 60 mg; riboflavin 250 mg; pyridoxine 60 mg; calcium panthotenate 240 mg; niacin 60 mg; folic acid 12 mg; biotin 50 mg; cyanocobalamine 4 mg

[c] Mineral mix:1 kg of diet contained $Ca(H_2PO_4)_2$ 15g; $MgSO_4$.$7H_2O$ 2.4g; KCl 4.5g; NaCl 2.1g; $FeSO_4.H_2O$ 155 mg; $CuSO_4$.$5H_2O$ 40 mg; $ZnSO_4$ .H_2O 80 mg; $MnSO_4.H_2O$ 30 mg; KI 11.7mg; $CoCl_2.6H_2O$ 4.8mg; Na_2SeO_3 2.4 mg

[d] Ascorbic acid phosphate ester (Purity >90%), Roche, China

[e] Tocopherol acetate (Purity 50%), Roche, China

[f] Soybean lecithin: contained 28% PE, 25% PC and 19% PI, with acetone insoluble>95%, American Soybean Association

2.2 Broodstock rearing

The experiment was conducted at the Tianjin Modern Fishery Technology and Engineering Center, Tianjin, China. In September 2004, pond-reared female crabs with an average individual body weight of 110 g (95-120 g) were obtained from Kunshan mitten crab farm, Jiangsu province, China and transported to the center. The average individual body weight was determined by randomly sampling 10 crabs after blotting. The hepatopancreas somatic index (HSI) and gonad somatic index (GSI) were determined as a percentage of the wet hepatopancreas and ovary weight on the crab body weight. Prior to the experiment, the crabs were acclimated for one week to the

indoor captive conditions and fed the control diet (Diet 1, without soybean lecithin supplementation). Crabs with immature gonads (GSI <1-2%) were selected in this experiment. Identification numbers were engraved on the carapace to facilitate monitoring of individual females. The crabs were randomly stocked into 5 indoor concrete tanks (with 8-m^2 bottom surface) at a density of 5 crab m^{-2}. Each tank was provided with approximate 10 cm of fine sand as bottom substrate for females to bury and extrude their eggs successfully, and 5 pieces of 30 cm long (8 inch diameter) PVC tubes served as shelter. For a period of approximate 7 months, female crabs were fed the selected experimental diets once daily at 18 hrs at a rate of 1-2% of the total body weight. Feces and excess feed were removed every morning. A constant water depth of 40 cm was maintained with 50% water exchanged every second day. The pH varied between 7.0 and 8.5, and the water temperature fluctuated from 20 ºC to 4 ºC according to the ambient temperature. Dissolved oxygen was kept at approximate 10 mg L^{-1}, while the ammonia and nitrite concentration was maintained below 0.5 mg L^{-1} and 0.2 mg L^{-1}, respectively.

2.3 Mating and spawning

Male crabs with an average body weight of 168 g (160-176 g) were obtained from Kunshan mitten crab farm in March, 2005. Before mating, the males were reared in an 8-m^2 fiber-glass tank containing freshwater and were fed fresh clam *(Sinonovacula constricta)* at a rate of 8-10% of the body weight. After one week acclimation, male crabs were transferred into the female culture tanks at a ratio of 3:1 (female:male) and the freshwater was replaced with 20 g L^{-1} diluted seawater in order to simulate their natural mating habitat (Zhang and Li, 2002). From then, berried crabs were identified and transferred into separate 150-L rectangle hatching tanks with 120 L diluted seawater (20 g L^{-1}) every second day in order to avoid disturbance by male crabs (each tank containing one berried female). Male crabs were removed from the culture tanks in order to keep a constant sex ratio. Berried females were then reared in these hatching tanks until the zoea 1 (Z1) larvae hatched. No sand was provided in the hatching tanks.

2.4 Evaluation criteria

2.4.1 First sampling

After 10 weeks rearing, 10 female crabs from each dietary treatment were randomly sampled for dissection. The wet body weight (WW) was recorded and ovaries and hepatopancreas were removed, weighed and stored at -70 ºC for biochemical analysis. GSI and hepatopancreas somatic index (HSI) were calculated using the following equations:

GSI (%) = 100 × Gonad WW /Body WW

HSI (%) = 100 × Hepatopancreas WW/Body WW

2.4.2 Second sampling

Mating occurred after about 6 month feeding, when the male crabs were introduced into the female rearing tanks in March, 2005. 18 days post-copulation, the spawning rate in each treatment was determined and calculated as follows:

Spawning rate =100×number of berried females/ (berried females + un-berried females)

Seven to ten berried crabs were sampled from each treatment 8-9 days after spawning. Excess water was removed by blotting, and female weight and egg clutch weight were recorded for each crab. Three egg sub-samples were weighted and the number of eggs was counted under a microscope (Nikon SMZ645) in order to estimate the total number of eggs in the clutch. The diameter of 100 eggs from each berried crab was measured under the microscope (Nikon YS100). Fecundity was determined as the number of eggs per female somatic weight (eggs g^{-1} female), and hatching rates were determined according to Cavalli et al. (1999) at a temperature and salinity of 22.0 ± 1.0 ºC and 20 g L^{-1}, respectively.

2.5 Chemical analysis

The experimental diets, gonad, hepatopancreas and egg samples were analyzed for proximate chemical composition, and/or fatty acid composition and lipid class composition. Moisture content and crude protein (Kjeldahl method, using a 6.25N as a

protein conversion factor) were analyzed following AOAC procedures (AOAC, 1985). Total lipid was determined according to Folch et al. (1957) as modified by Ways and Hanahan (1964), and total lipids fatty acid methyl ester (FAME) composition was analytically verified by gas chromatography following the methodology of Coutteau and Sorgeloos (1995). Lipid classes were analyzed using high-performance-thin-layer chromatography (HPTLC) (Olsen and Henderson, 1989).

2.6 Statistical analysis

Data were presented as mean ± standard deviation and were subjected to statistical analysis using the software SPSS (version 11.0). Except for the differences in total lipid and moisture content of hepatopancreas and ovary between initial values obtained before starting the feeding experiment and values after 10 weeks feeding, which were examined by Student's t-test, statistical significance of differences for the other parameters were determined using one way ANOVA. Homogeneity of variance was tested with Levene's test and data were arcsine-square root or logarithmic transformed when necessary. Tukey's Unequal Number HSD was applied to detect significant differences between means. When a normal distribution and/or homogeneity of the variances could not be achieved, data were subjected to the Kruskal-Wallis H nonparametric test, followed by the Games-Howell nonparametric multiple comparison test (Sokal and Rohlf，1995). $P<0.05$ was regarded statistically significantly different.

3. Results

3.1 Chemical composition of experimental diets

The analyzed proximate composition, lipid class and fatty acid composition of the diets was well within the desired levels (Table 2). The levels of protein, total lipid and total n-3 HUFA (≥20:3n-3) were similar among the different experimental diets. And as expected, the levels of total PL, phosphatidylethanolamine and phosphatidic acids (PE+PA), phosphatidylserine and phosphatidylinositol (PS+PI), and phosphatidylcholine (PC) were significantly different.

3.2 Effect of dietary soybean lecithin on GSI, HSI of females

After 10 weeks feeding, the HSI decreased from 10.3% to approximate 6.3%, no significant differences were observed among the treatments (Fig. 1). The GSI increased dramatically from 1.8% to 8.4-10.6% for all treatments, and the females fed Diet 3 (2.4% soybean lecithin supplementation) had a significantly higher GSI than the females fed Diet 1 and Diet 2 (0% and 1.2% soybean lecithin supplementation, respectively) ($P<0.05$), while GSI of the females fed Diet 4 was in between.

Table 2. Protein and total lipid content, and phospholipid and fatty acid composition of the experimental diets supplemented with different levels of soybean lecithin

	Diet 1(0%)	Diet 2 (1.2%)	Diet 3 (2.4%)	Diet 4 (3.6%)
Protein (% dw)	40.3	40.6	40.5	41.1
Lipid (% dw)	8.3	8.7	8.9	8.5
Total PL (% dw)	0.3	1.3	2.4	3.2
PE+PA	0.2	0.5	0.8	1.1
PS+PI	0	0.3	0.5	0.7
PC	0	0.3	0.6	1.0
Other PL	0.2	0.3	0.5	0.5
Fatty acids (mg g^{-1} dw)				
14:0	1.5	1.2	1.1	1.1
16:0	10.6	10.2	10.0	10.1
18:0	4.4	4.1	3.7	3.5
18:1 (n-9)	14.6	11.9	9.5	7.7
18:1 (n-7)	1.1	1.0	0.7	0.6
18:2 (n-6)-c	26.9	24.0	20.8	18.7
18:3 (n-3)	3.2	3.0	2.6	2.2
18:4 (n-3)	0.1	0.1	0.2	0.2
20:0	0.5	0.4	0.4	0.3
20:1 (n-9)	1.2	0.9	0.8	0.8
20:4 (n-6)	0.4	0.4	0.5	0.5
20:4 (n-3)	0.4	0.5	0.5	0.5
20:5 (n-3)	6.3	7.4	7.6	7.2
21:5 (n-3)	0.5	0.1	0.6	0.6
22:4 (n-6)	0.1	0.1	0.1	0.1
22:5 (n-3)	1.6	1.7	1.8	1.7
22:6 (n-3)	6.8	7.4	7.8	7.3
∑ Saturates	17.0	15.9	15.2	15.0
∑ Mono-unsaturates	15.7	12.9	10.2	8.3
∑ n-6 PUFA (≥18:2n-6)	27.4	24.5	21.4	19.3
∑ n-3 HUFA (≥20:3n-3)	16.0	17.2	18.4	17.4

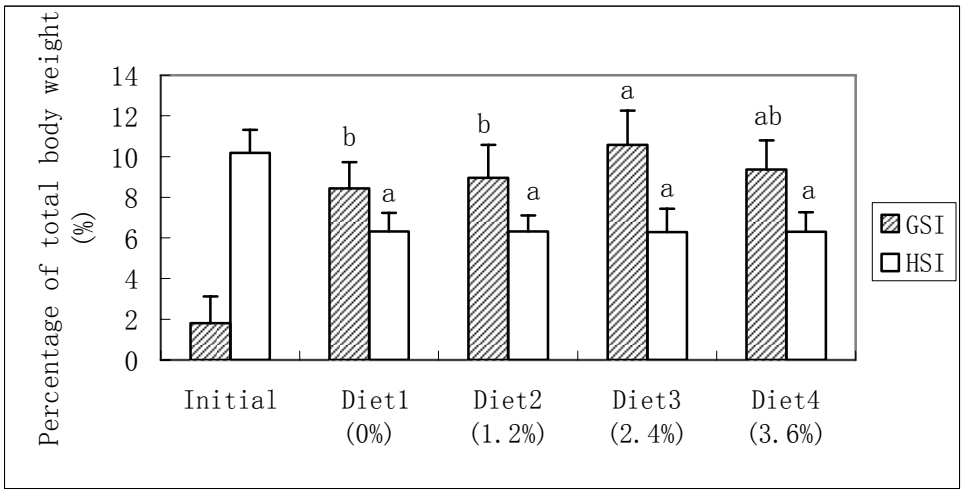

Fig. 1. Gonad somatic index (GSI) and hepatopancreas somatic index (HSI) of *E. sinensis* fed diets supplemented with different levels of soybean lecithin for 10 weeks. Data were presented as mean ± standard deviation (n=10). Bars with the same superscript are not statistically significantly different (P>0.05)

3.3 Effect of dietary soybean lecithin on moisture and total lipid content of hepatopancreas and gonad, and lipid class composition of eggs

After 10 weeks feeding, females fed Diet 2 had a significantly higher hepatic dry matter content and lower hepatic lipid content than females fed any of the other diets (P<0.05), but no significant difference was observed for the two parameters in the ovary among the other treatments (Table 3). Nevertheless, compared to the initial values, the hepatic dry matter content increased significantly from 41.6% to 51.4-58.3%, and ovarian dry matter content decreased significantly from 56.7% to 50.9-51.3% after the females were fed the experimental diets for 10 weeks (P<0.05). An inverse relationship was found for the lipid content in these two tissues.

Total lipid content and lipid class composition in eggs are presented in Table 4. Total lipid content of eggs ranged from 29.2% to 35.3% of egg dry weight; higher lipid content was found in eggs from the females fed Diet 4 (3.6% soybean lecithin supplementation) (P<0.05). Neutral lipids (NL), mainly triglycerides (TG) were predominant in the eggs, comprising about 20% of egg dry weight, and no significant difference was observed in any of the NL components among the treatments, except for the higher free fatty acids level in the eggs of females fed Diet 4. Total PL content of

eggs ranged from 8.4% to 10.9% of egg dry weight, and was significantly lower in the eggs of females fed Diet 1 than that of females fed any other diet (P<0.05). PC was the main component of total PL, comprising about 7-8% in the egg dry weight, but no significant difference could be found among treatments.

Table 3. Dry matter content and total lipid content in hepatopancreas and ovary of *E. sinensis* fed diets supplemented with different levels of soybean lecithin for 10 weeks

	Dry matter content (%)		Total lipid content (% WW)	
	Hepatopancreas	Ovary	Hepatopancreas	Ovary
Initial sampling (in September)	41.6±6.5 ^{B}	56.7±5.5 ^{A}	45.9±7.2 ^{A}	13.2±2.8 ^{B}
First sampling (in December)				
Diet 1 (0%)	51.4 ± 5.9 $^{A\,b}$	51.3 ± 2.0 ^{B}	35.0 ± 7.7 $^{B\,a}$	16.1 ± 1.3 ^{A}
Diet 2 (1.2%)	58.3 ± 7.8 $^{A\,a}$	51.0 ± 1.2 ^{B}	25.9 ± 5.5 $^{B\,b}$	16.0 ± 0.9 ^{A}
Diet 3 (2.4%)	53.4 ± 7.0 $^{A\,ab}$	50.9 ± 3.3 ^{B}	32.7 ± 9.7 $^{B\,ab}$	15.4 ± 1.6 ^{A}
Diet 4 (3.6%)	53.9 ± 5.3 $^{A\,ab}$	51.1± 0.9 ^{B}	31.1 ± 7.1 $^{B\,ab}$	15.4 ± 0.9 ^{A}

Data were presented as mean ± standard deviation (n=10). Values in the same column that do not share the same superscript are statistically significantly different (P<0.05). Capital letters represent the statistical analysis result including the initial values obtained before starting the feeding experiment (using Student's t-test); small letters represent the statistical analysis result without initial values (using one way ANOVA)

3.4 Effect of dietary soybean lecithin on broodstock reproductive performance and egg quality

Higher spawning percentages were observed for the females fed Diet 4 and Diet 3 (95% and 92%, respectively) than the females fed Diet 2 and Diet 1 (both 81%) (Table 5). Egg production (total number of eggs female^{-1}) and fecundity increased with increasing dietary soybean lecithin levels, ranging from 30.8×10^4 to 41.1×10^4 eggs female^{-1} and 2957 to 4106 eggs g^{-1} female, respectively. Females fed Diet 1 and Diet 2 had a significantly lower egg production and fecundity than the groups fed Diet 4, and the females fed Diet 3 had intermediate values (P<0.05). No significant differences in egg diameter were observed.

Table 4. Total lipid content and lipid class composition of eggs produced by female *E. sinensis* fed diets supplemented with different levels of soybean lecithin for 7 months

Treatments (Number of samples)	Diet 1 (0%) (8)	Diet 2 (1.2%) (9)	Diet 3 (2.4%) (7)	Diet 4 (3.6%) (7)
Total lipid (% WW)	11.0 ± 1.3 ^{b}	11.1 ± 1.3 ^{b}	10.3 ± 1.0 ^{b}	13.1 ± 1.2 ^{a}
Total lipid (% dw)	30.4 ± 2.7 ^{b}	30.7 ± 2.8 ^{b}	29.2 ± 2.1 ^{b}	35.3 ± 4.4 ^{a}
Total NL (% dw)	21.8 ± 1.8	20.6 ± 2.7	20.5 ± 1.9	22.9 ± 2.8
Cholesterolesters	0.37 ± 0.14	0.34 ± 0.08	0.26 ± 0.11	0.39 ± 0.10
Triglycerides	20.43 ± 1.89	19.06 ± 2.66	19.0 8 ± 1.92	20.85 ± 3.08
Free fatty acids	0.14 ± 0.04 ^{a}	0.03 ± 0.04 ^{b}	0.06 ± 0.02 ^{ab}	0.18 ± 0.20 ^{a}
Cholesterol + Diacylglycerol	0.80 ± 0.04	1.04 ± 0.39	0.98 ± 0.16	1.25 ± 0.25
Total PL (% dw)	8.4 ± 1.2 ^{b}	9.0 ± 1.1 ^{ab}	8.8 ± 1.2 ^{ab}	10.9 ± 1.9 ^{a}
PA+PE	0.86 ± 0.35 ^{b}	1.17 ± 0.38 ^{ab}	1.03 ± 0.25 ^{ab}	1.41 ± 0.36 ^{a}
PS+PI	-	0.08 ± 0.13	0.05 ± 0.06	0.19 ± 0.27
PC	7.00 ± 1.27	7.36 ± 0.77	7.25 ± 1.07	8.76 ± 1.59
SPM	0.11 ± 0.06	0.13 ± 0.03	0.14 ± 0.04	0.14 ± 0.02
Lyso PC	-	-	-	0.03 ± 0.05

PL=phospholipids, PA=phosphatidic acids, PE=phosphatidylethanolamine, PS=phosphatidylserine, PI=phophatidylinositol, PC=phosphatidylcholine, SPM=fingomyelines, Lyso PC=Lyso-phosphatidylcholine. Data were presented as mean ± standard deviation. Values in the same line that do not share the same superscript are statistically significantly different (P <0.05)

Table 5. Spawning percentage, body weight, total egg production per female, fecundity, egg diameter and hatching rate of *E. sinensis* fed diets supplemented with different levels of soybean lecithin for 7 months

Treatments (No. of samples)	Diet 1 (0%) (8)	Diet 2 (1.2%) (7)	Diet 3 (2.4%) (8)	Diet 4 (3.6%) (10)
Body weight (g)	117 ± 9	113 ± 6	119 ± 9	117 ±6
Spawning percentage (%)	81	81	92	95
Total No. of eggs per female ($\times 10^4$)	30.8 ± 4.7^{b}	33.9 ± 4.9^{b}	39.4 ± 5.5^{ab}	41.1 ± 5.8 ^{a}
Fecundity (Number of eggs g^{-1} female)	2957 ± 39^{c}	3312 ± 49^{bc}	3825 ± 51^{ab}	4106 ± 73^{a}
Egg diameter (μm)	351 ± 14	343 ± 9	345 ± 4	342 ± 15
Egg incubation time (day)	21.1 ± 0.5^{b}	19.8 ± 0.4^{a}	21.3 ± 0.4^{b}	20.9 ± 1.1^{b}
Egg hatching rate (%)	54	55	51	50

Data were presented as mean ± standard deviation. Values in the same column that do not share the same superscript are statistically significantly different (P<0.05). The initial value of average crab body weight before starting feeding experiment was 110 g

After about 7 months feeding, the average crab body weight increased from 110 g to

113-119 g in different treatments, no significant difference was obtained among treatments. Eggs needed minimum of 18 days incubation at 22 ±1.0 ºC to start hatching. The eggs from the females fed Diet 2 had a significantly shorter hatching period (19.8 day) than eggs from the females fed any of other diets (20.9 to 21.3 day) (P<0.05) (Table 5). No remarkable differences were observed in egg hatching rate among any of the treatments at the end of incubation period, which ranged from 50% to 55%.

4. Discussion

In nature, mitten crab accumulates energy and develops their reproductive organs during their catadromous migration in a period of August to November. Mating occurs from December to March, during which final ovarian maturation is achieved (Xue et al., 1987). Our study demonstrated that, after 3 months feeding, the ovarian lipid content increased from 13.2% to 15.4-16.1% of the dry weight, with a 4-5 fold increase in GSI from 1-2% to 9-10%; whilst the hepatic lipid content decreased from 45.9% to 25.9-35.0%, with a concomitant decrease of HSI from 10% to 5-6%. These results are in line with the observations by Cheng et al. (1998a; 2000a) and Wen et al. (2001), demonstrating the lipid mobilization from the hepatopancreas to the gonad during the reproductive maturation of *E. sinensis* broodstock.

Previous studies indicated that lipid mobilization to the developing ovary depends much more on the ingestion of dietary lipids than on lipids stored in the midgut gland (hepatopancreas) (Harrison, 1990; Cavalli et al., 2001). In this study, however, a relatively high PL level (8.4% of dw) was recorded in eggs from females fed Diet 1, which was very low in PL (0.3% of dw). This seems to indicate that not only the dietary PL, but also the hepatic PL reserves are transported to the developing ovaries as reported earlier by Cheng et al. (1998a) and Wen et al. (2001). It may also be that triglycerides (TG) reserves are converted to PL and transported to the ovary as observed by Teshima and Kanazawa (1980). This could however not be confirmed here as no lipid class composition of hepatopancreas was recorded. On the other hand, the higher dry matter content, but lower total lipid level in the hepatopancreas in the females fed

Diet 2 is however difficult to explain. Overall, the sharp increase in egg PL levels regardless of the dietary PL level indicates the importance of PL in the ovarian development of *E. sinensis*.

In a study on broodstock of *Marsupenaeus japonicus*, Alava et al. (1993) showed that dietary PL influence the mobilization of lipids from the hepatopancreas to the ovary, and higher dietary PL result in higher GSI. Meanwhile, Cheng et al. (2000b) found that ovaries of female crabs that did not spawn within 14 days post-copulation contained less PL and HUFA than those of normal spawning crabs (>90% crabs spawned within 7 days after copulation) and suggested that PL may facilitate transport of lipids to the ovary and accelerate ovarian maturation and spawning of *E. sinensis*. In the present study, females fed Diet 3 had significantly higher GSI than females fed Diet 1 and Diet 2, which is in accordance with the results of previous studies.

Our results showed that spawning percentage was higher for the females fed Diet 3 and Diet 4. Meanwhile the total numbers of eggs as well as the fecundity were also improved with increasing dietary PL. These results are in line with the earlier studies on penaeid shrimps (Millamena et al., 1986; Bray et al., 1989; Cahu et al., 1994) and mitten crabs (Wu et al., 2007d). In this study, however, the egg hatching rates among the treatments were not significantly different as observed by Cahu et al. (1994) and Wu et al. (2007d). It could be explained by the *in vitro* egg hatching rate determination used in the current study, for which the egg hatching is very much influenced by hatching conditions such as improper aeration and fungi infection (Bao et al., 1999). On the other hand, it remains also unclear why egg incubation time was significantly shorter for the females fed Diet 2.

In conclusion, the positive effects of dietary PL supplementation for mitten crab broodstock rearing were demonstrated. Exact requirement and the impact on larval quality should be further studied.

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Chapter IV Section II

Effect of dietary HUFA on tissue fatty acid composition and reproductive performance of Chinese mitten crab *Eriocheir sinensis* broodstock

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Abstract

This study was conducted to determine the optimum highly unsaturated fatty acid (HUFA) level in semi-purified diets for female Chinese mitten crab *Eriocheir sinensis* broodstock. Five isolipidic and isonitrogenous diets were formulated to contain different HUFA levels (0.14, 0.65, 1.15, 1.64 and 2.07%) by the supplementation of HUFA-rich fish oil. Diets were fed to triplicate groups of 25 female crabs (75 animals per treatment) stocked in concrete tanks for a period of 8 months. After 6 month feeding, male crabs were introduced into the tanks and, mating, spawning and egg hatching occurred in brackish water in the following month. The nutritional value of the different formulated diets was assessed based on tissue fatty acid composition, gonad somatic index (GSI), spawning percentage, egg production per female, fecundity (eggs g^{-1} female weight), and egg quality parameters (fatty acid composition and egg diameter).

There were no significant differences in GSI, hepatopancreas somatic index (HSI), and total ovarian and hepatic lipid content between the treatments after 3 months feeding the different diets. Also reproductive performance was similar for all treatments. Females fed the diets containing higher HUFA levels only tended to spawn earlier. Furthermore, a significant positive correlation was observed between the hepatopancreas, ovary and egg HUFA levels and the dietary HUFA content; however, no significant correlation could be found between muscle HUFA level and dietary HUFA content. The fact that the diets containing 1.64 and 2.07 % HUFA resulted in similar HUFA levels in hepatopancreas, ovary and eggs, could indicate that a total HUFA level of 1.64% of the diet dry weight is sufficient for *Eriocheir sinensis* broodstock diets. The effect of dietary HUFA content on offspring quality should however be further investigated.

Keywords *Eriocheir sinensis*; broodstock; highly unsaturated fatty acids (HUFA); fatty acid composition; reproductive performance

1. Introduction

Chinese mitten crab *Eriocheir sinensis* is a popular food in China because of its high meat yield, delicate flavor and high nutritional value, so its market demand and the price are very high (Zhang and Li, 2002； Chen et al., 2007). For this reason, mitten crab seed is widely cultured and stocked in ponds, reservoirs, and lakes. The last two decades hatchery techniques have been developed successfully. However, the highly variable quality of eggs is still a bottleneck for the reliable production of larvae and postlarvae in many commercial hatcheries.

The nutritional status of crustacean broodstock is of utmost importance for reproduction and offspring quality and unbalanced or incomplete diet cause poor reproductive performance or may even stop animals from reproducing (Harrison et al., 1990; Bray and Lawrence, 1992; Wouters et al., 2001a). Hence, a better understanding of the nutritional requirements of *Eriocheir sinensis* broodstock could lead to improved hatchery output both quantitatively and qualitatively. Crustaceans have been recognized as having limited ability to synthesize highly unsaturated fatty acids *de novo* (Kanazawa et al., 1979; Mourente, 1996). Several studies have shown that dietary HUFA have a positive effect on ovarian maturation, reproductive performance, and egg and larvae quality of penaeid shrimp (Millamena, 1989; Alava et al., 1993；Cahu et al., 1994；Xu et al., 1994； Cahu et al., 1995; Wouters, et al., 1999; 2001c) and the giant freshwater prawn *Macrobrachium rosenbergii* (Cavalli et al., 1999).

Although previous studies have shown it is possible to completely replace fresh food with formulated diets for *Eriocheir sinensis* broodstock, limited information is available on the lipid requirements of *Eriocheir sinensis* broodstock (Wen et al., 2002c; Wu, 2004; Wu et al., 2007d). A number of publications showed the changes in lipid composition (total lipid level, lipid class composition and fatty acid profile) of the hepatopancreas, ovary and eggs during ovary maturation of *Eriocheir sinensis* broodstock (Cheng et al., 1998a; 1998b; 1999; 2000a; 2000b; Wen et al., 2001; Ying et al., 2004; 2006). Moreover, Wen et al. (2002c) studied ovarian maturation and reproductive performance when

including different lipid sources (anchovy oil, soybean oil and pork lard) into the *Eriocheir sinensis* broodstock diet and found the diet containing anchovy oil resulted in the highest n-3 HUFA level in the eggs, and the highest fecundity and hatchability. Wu et al. (2007d) further reported higher gonad somatic index (GSI) and hepatopancreas somatic index (HSI), and a better fecundity, egg hatching rate and larval quality in terms of carapace length and stress resistance of pond-reared *Eriocheir sinensis* broodstock fed diets supplemented with phospholipids, HUFA, cholesterol, vitamin C and vitamin E. To our best knowledge, the optimal HUFA level in a formulated diet for *Eriocheir sinensis* broodstock has however not yet been studied, which will be a hindrance to develop cost-effective and reliable formulated broodstock diets.

The aim of this study was to determine the optimal HUFA level in a formulated diet for *Eriocheir sinensis* broodstock. Five different levels of HUFA were formulated into the semi-purified diets, and GSI, HSI, tissue lipid composition, spawning percentage, egg production and fecundity, as well as a number of egg characteristics (fatty acid composition and egg diameter) were used to evaluate the optimum dietary HUFA level.

2. Materials and Methods

2.1 Experimental diets

Five compound diets were formulated to contain different levels of HUFA. The constant and variable ingredients of the experimental diets are shown in Table 1. In order to maintain the same total lipid level but different HUFA levels in the diets, the relative proportions of HUFA-rich fish oil and HUFA free hydrogenated plant oil were altered. Soybean oil was added into the diets in order to obtain proper level of n-6 fatty acids. The dry ingredients were thoroughly mixed and lipid ingredients were added drop by drop to the dry mixture while blending. All ingredients were mixed with 20% (w/w) hot water. The resulting dough was extruded through a 4-mm orifice die and dried with the aid of a ventilator at room temperature for 72 h. The resulting experimental diets were stored at -20 °C for later use.

Table 1. Formulation of the experimental diets with different levels of HUFA supplementation

Constant ingredients	%				
Casein	30.0				
Crude corn starch	28.75				
Gelatin	15.0				
Defatted soybean protein isolate	10.0				
Yeast extract	0.7				
Soybean lecithin [a]	2.0				
Cholesterol	0.5				
Sodium alginate	1.5				
Vitamin mix [b]	1.0				
Mineral mix [c]	3.0				
Choline chloride	1.0				
Inositol	0.6				
Ascorbic acid phosphate ester [d]	0.3				
Tocopherol acetate [e]	0.1				
Butylated hydroxytoluene (BHT)	0.01				
Soybean oil	1.5				
Variable ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Hydrogenated plant oil	4.0	3.0	2.0	1.0	0
Fish oil [d] (HUFA content)[f]	0 (0)	1.0 (0.6)	2.0 (1.2)	3.0 (1.8)	4.0 (2.4)

[a] Soybean lecithin: contained 28% PE, 25% PC and 19% PI, with acetone insoluble>95%, American Soybean Association

[b] Vitamin mix: 1 kg of diet contained vitamin A 10,000 IU; vitamin D 2,500 IU; vitamin K 64 mg; thiamin 60 mg; riboflavin 250 mg; pyridoxine 60 mg; calcium panthothenic 240 mg; niacin 60 mg; folic acid 12 mg; biotin 50 mg; cyanocobalamine 4 mg

[c] Mineral mix:1 kg of diet contained $Ca(H_2PO_4)_2$ 15g; $MgSO_4$.$7H_2O$ 2.4g; KCl 4.5g; NaCl 2.1g; $FeSO_4.H_2O$ 155 mg; $CuSO_4$.$5H_2O$ 40 mg; $ZnSO_4$ .H_2O 80 mg; $MnSO_4.H_2O$ 30 mg; KI 11.7mg; $CoCl_2.6H_2O$ 4.8mg; Na_2SeO_3 2.4 mg

[d] Ascorbic acid phosphate ester (Purity >90%), Roche, China

[e] Tocopherol acetate (Purity 50%), Roche, China

[f] Fish oil: contained 60% HUFA, DHA/EPA = 1; Chemport Co., Ltd, Korea

2.2 Source of broodstock and culture conditions

The experiment was conducted at the Tianjin Modern Fishery Technology and Engineering Center, Tianjin, China. In August 2006, pond-reared female crabs with an average body weight of 111 g (90-120 g) and immature gonads (the average GSI < 1%), were obtained from the Kunshan mitten crab farm, Jiangsu province, China and transported to the center. Upon arrival, 10 crabs were randomly sampled and the tissue of hepatopancreas and gonad were dissected and preserved at -70°C for later biochemical analysis. Prior to the start of the experiment, the crabs were acclimated to indoor captive conditions for one week when they were fed the control diet (Diet 1,

without HUFA supplementation). Identification numbers were engraved on the carapace of the animals to facilitate monitoring of individual females. The crabs were randomly stocked into 15 indoor concrete tanks (with 6-m^2 bottom surface) at a density of 4 crab m^{-2}. Three replicates were conducted for each dietary treatment and each replicate contained 25 crabs. The tank bottoms were covered with an approximate 10 cm thick layer of fine sand as substrate for the females to bury and attach their eggs to their abdomens. Five pieces of 30 cm long (8 inch diameter) PVC tubes were also placed on the bottom of each tank to serve as shelters. For a period of approximate 8 months, female crabs were fed the experimental diets once daily at 18 hrs at a rate of 1-3% of the total body weight depending on the water temperature. Faeces and excess feed were removed every morning. A constant water depth of 40 cm was maintained with 50% water exchanged every second day. The pH fluctuated between 7.0 to 8.5, and the water temperature dropped from 21°C to 5°C, and then increased to 18°C according to the ambient temperature. Dissolved oxygen was kept at approximate 10 mg L^{-1}, while both ammonia and nitrite concentration were maintained below 0.2 mg L^{-1}.

Male crabs with an average body weight of 144 g (138-156 g) were obtained from the same crab farm in March 2007. Before mating, the males were reared in an 8-m^2 fiber-glass tank containing freshwater and were fed fresh razor clam (*Sinonovacula constricta*) at a rate of 8-10% of the total body weight.

2.3 Mating and spawning

On March 12, 2007, male crabs were transferred into the female culture tanks at a sex ratio of 2:1 (female: male). At the same time, the salinity of the rearing water was gradually increased to 20 g L^{-1} in order to stimulate mating. From then onwards, every second day, berried crabs were identified and transferred into separate 150-L rectangle hatching tanks containing 120 L diluted seawater (20 g L^{-1}) in order to avoid disturbance by male crabs. Male crabs were removed from the culture tanks in order to keep a constant female/male ratio. The berried females were then reared individually in these hatching tanks until the zoea 1 (Z1) larvae hatched.

2.3 Evaluation criteria

2.3.1 GSI and HSI of broodstock

After 3 months rearing, 10 female crabs from each dietary treatment (3 or 4 females from each replicate) were randomly sampled for dissection. The wet body weight of the crab (BW) was recorded and ovaries, hepatopancreas and muscle were removed, weighed and stored at -70 °C for biochemical analysis. GSI and HSI were calculated using the following equations:

GSI (%) = 100 × gonad WW /BW

HSI (%) = 100 × hepatopancreas WW /BW

2.3.2 Reproductive performance

Mating occurred after about 7 month feeding, when the male crabs were introduced into the female rearing tanks. The spawning percentage in each treatment was determined at 18 days post-copulation and calculated as follows:

Spawning percentage=100×number of berried females/(berried females + un-berried females)

Meanwhile, 10 berried crabs were sampled from each treatment (3 or 4 females from each replicate) 8-9 days after spawning. Excess water was removed by blotting, and the body weight and the weight of the egg mass were recorded for each crab. Three egg sub-samples (approximate 0.010 g) were weighted and counted under a microscope (Nikon SMZ645) and the total number of eggs was estimated. The diameter of 100 eggs from each berried crab was measured under the microscope (Nikon YS100). Fecundity was estimated as the number of eggs per female somatic weight (eggs g^{-1} female). Egg mass was preserved at -70 °C for biochemical analysis.

2.4 Biochemical analysis

The total lipid content of the experimental diets and the samples of gonad, hepatopancreas were determined according to Folch et al. (1957) as modified by Ways and Hanahan (1964). The fatty acid composition of the diets, ovary, hepatopancreas,

muscle and eggs were analytically verified by gas chromatography following the methodology of Coutteau and Sorgeloos (1995).

2.5 Statistical analysis

Data are presented as mean ± standard deviation. Statistic analyses were determined using the software SPSS (version 12.0). Homogeneity of variances was tested with the Levene's test, using arcsine-square root or logarithmic transformation when necessary. Statistical analyses were conducted using one way analysis of variance (ANOVA) and compared with Tukey-b (for unequal number of replicates) as post-hoc test. When the homogeneity of the variances could not be achieved, data were subjected to the Kruskal-Wallis H nonparametric test. The linear regression correlations between the HUFA levels in the diets and in the hepatopancreas, ovaries, muscles and eggs of bloodstock were analyzed using General Regression Models. $P<0.05$ was regarded statistically significantly different or significant positive correlation.

3. Results

3.1 HUFA profile of the experimental diets

The fatty acid composition of the experimental diets was well within the desired levels (Table 2). As expected, the total PUFA (polyunsaturated fatty acid, C≥18:2n) and HUFA (C≥20:3n) level in the experimental diets increased with increasing HUFA-rich fish oil supplementation. Diet 1, 2, 3, 4 and 5 contained 19.9, 21.4, 28.7, 30.7 and 35.5 mg g^{-1} dw (dry weight) PUFA, and 1.4, 6.5, 11.5, 16.4 and 20.7 mg g^{-1} dw HUFA, respectively. As a consequence, the total SFA (saturated fatty acid) content decreased gradually from 51.7 mg g^{-1} dw in Diet 1 to 17.9 mg g^{-1} dw in Diet 5. Meanwhile the total MUFA (mono-unsaturated fatty acid) content remained constant, ranging from 7.1 to 9.0 mg g^{-1} dw. The moisture content of the diets ranged from 10 to 11.6%, and total lipid content ranged from 10.0 to 10.9% of the dry weight.

Table 2. Moisture content, total lipid content and fatty acid composition of the experimental diets with different levels of HUFA supplementation

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Moisture content (%)	10.6	11.1	10.6	11.6	10.2
Total lipid content (% dw)	10.2	10.9	10.1	10.0	10.9
Fatty acids (mg g^{-1} dw)					
16:0	33.5	26.8	22.3	18.7	12.1
17:0	0.3	0.3	0.3	0.3	0.2
18:0	17.8	14.5	12.8	9.4	5.6
18:1n-9+n-7	9.0	7.7	8.3	7.7	7.1
18:2n-6	16.1	13.0	15.1	13.4	13.0
18:3n-3	2.4	1.9	2.1	1.9	1.8
20:4n-6 (ARA)	0.3	1.1	0.9	1.5	1.8
20:5n-3 (EPA)	0.6	2.9	5.4	7.6	9.7
22:6n-3 (DHA)	0.5	2.5	5.2	7.3	9.2
∑ SFA	51.7	41.6	35.5	28.4	17.9
∑ MUFA	9.0	7.7	8.3	7.7	7.1
∑ PUFA (C≥18:2n)	19.9	21.4	28.7	30.7	35.5
∑ n-3 PUFA	3.5	7.3	12.7	16.8	20.7
∑ n-6 PUFA	16.4	14.1	16.0	13.9	14.8
n-3/ n-6	0.2	0.5	0.8	1.2	1.4
∑HUFA (C≥20:3n)	1.4	6.5	11.5	16.4	20.7
∑ HUFA (%)	0.14	0.65	1.15	1.64	2.07
DHA/EPA	0.83	0.86	0.96	0.96	0.95

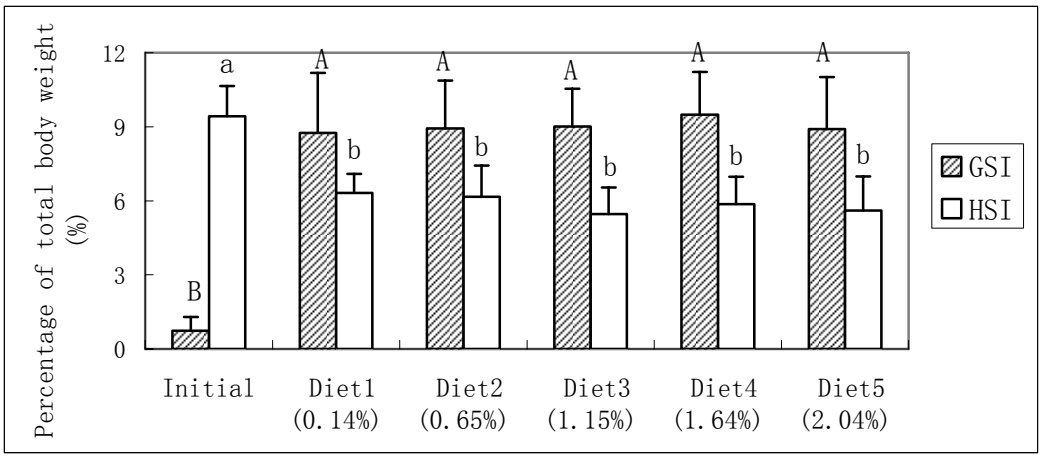

Fig. 1. Gonad somatic index (GSI) and hepatopancreas somatic index (HSI) of *Eriocheir sinensis* broodstock at the start of the experiments and after being fed the experimental diets containing different HUFA levels for 3 months. Data were presented as mean ± standard deviation (n=10). Values with different letters are significantly different (P<0.05). Capital letters refer GSI; small letters refer to HSI

Table 3. Dry matter content and total lipid content of hepatopancreas and ovary of *Eriocheir sinensis* broodstock fed the experimental diets containing different HUFA levels for 3 months

	Dry matter content (%)		Total lipid content (%WW)	
	Hepatopancreas	Ovary	Hepatopancreas	Ovary
Initial (in September)	54.1 ± 5.0	26.6 ± 6.3^{A}	45.4 ± 5.3^{A}	5.8 ± 2.1^{A}
First sampling (in December)				
Diet 1 (0.14%)	47.8 ± 7.6	43.9 ± 4.5$^{B\ b}$	34.9 ± 8.1^{B}	13.9 ± 1.7^{B}
Diet 2 (0.65%)	49.8± 6.7	48.1 ± 2.5$^{B\ a}$	35.8± 5.9^{B}	12.9 ± 1.0^{B}
Diet 3 (1.15%)	47.0 ± 7.4	45.5 ± 3.7$^{B\ ab}$	35.0 ± 7.8^{B}	14.1 ± 1.6^{B}
Diet 4 (1.64%)	45.5 ± 7.9	46.4± 2.1$^{B\ ab}$	32.5 ± 4.5^{B}	13.6 ± 1.4^{B}
Diet 5 (2.07%)	45.9± 7.0	46.2± 1.7$^{B\ ab}$	30.6± 5.3^{B}	13.8 ± 1.0^{B}

Data were presented as mean ± standard deviation (n=10). Values in the same column that do not share the same superscript are statistically significantly different (P<0.05). Capital letters represent statistical analysis results including the initial value obtained before starting he experiment; small letters represent statistical analysis results without the initial value

3.2 Effect of dietary HUFA on GSI, HSI, dry matter content and total lipid content of hepatopancreas and ovary

After feeding the crabs for 3 months with the experimental diets, the HSI of the crabs decreased significantly from the initial value of 9.4 to 5.5-6.3%, with a concomitant increase of the GSI from 0.7 to 8.8-9.5% in all treatments (Fig. 1). No significant differences in HSI and GSI were found among the treatments (P>0.05). Compared to the initial values, the hepatic dry matter content decreased slightly from 51.4 to 45.5-49.8%, but ovarian dry matter content increased significantly from 26.6 to 43.9-48.1% (P<0.05). Meanwhile, the total hepatic lipid content decreased significantly from 45.4 to 30.6-35.8% while the total ovarian lipid content increased significantly from 5.8 to 12.9-14.1% (P<0.05) (Table 3). Nevertheless, no significant difference were observed for the ovarian dry matter content, total ovarian and hepatic lipid content among the treatments, except for the females fed Diet 2, which had a significantly higher ovarian dry matter content than those fed Diet 1 (P<0.05).

3.3 Effect of dietary HUFA on fatty acids composition of hepatopancreas, ovary, muscles and eggs

The fatty acid composition of hepatopancreas and ovary at the start of the experiment is shown in Table 4 and Table 5. Together with a higher total lipid level (Table 3),

hepatopancreas contained more total SFA, MUFA and PUFA compared to ovary. Total HUFA levels were however similar in hepatopancreas and ovary (27.0 and 28.4 mg g^{-1} dw, respectively), while hepatopancreas had a higher DHA/EPA ratio than ovary (1.5 and 1.2, respectively).

The fatty acid composition of the hepatopancreas, ovary and muscle of crabs fed the experimental diets for 3 months as well as freshly-spawned eggs of the different treatments are shown in Table 4, 5, 6 and 7. In general, the predominant fatty acids in the hepatopancreas, ovary, muscle and eggs were 16:0, 16:1n-7, 18:1n-9+n-7, 18:2n-6, 18:3n-6, 18:3n-3, 20:4n-6 (ARA), 20:5n-3 (EPA) and 22:6n-3 (DHA), while hepatopancreas had a higher DHA/EPA (0.9-1.2) compared to ovary (0.7-0.8), muscle (0.6-0.7) and eggs (0.7-0.8). The EPA, DHA and total HUFA content of the hepatopancreas, ovary and eggs was greatly influence by the dietary HUFA level. Significant positive correlations were recorded between the total HUFA level in the hepatopancreas, ovary and egg and the dietary HUFA content (Fig. 2A, Fig. 2B and Fig. 2D, n=5, $P<0.05$). However, no significant correlation could be found between the total HUFA level in muscle and the dietary HUFA content (Fig. 2C, n=5, $P=0.37$). Along with the increasing dietary HUFA level, no significant difference could be found in the ovarian EPA, DHA and total HUFA content of crabs fed Diets 3, 4 and 5, as well as the egg EPA, DHA and HUFA level of crabs fed Diets 4 and 5.

Table 4. Fatty acid composition of hepatopancreas of *Eriocheir sinensis* broodstock at the start of the experiment and after being fed the experimental diets containing different HUFA levels for 3 months

Fatty acids (mg g^{-1} dw)	Initial	Diet 1 (0.14%)	Diet 2 (0.65%)	Diet 3 (1.15%)	Diet 4 (1.64%)	Diet 5 (2.07%)
16:0	64.8	44.6±4.4	54.7±9.3	50.7±8.1	47.6±9.2	46.7±9.6
16:1 n7	52.7	33.3±10.8^{ab}	45.5±8.1^{a}	39.3±10.6^{ab}	35.3±9.5^{ab}	30.0±9.0^{b}
17:1 n7	-	2.0±0.8	2.3±0.7	2.6±0.9	2.1±0.5	2.1±0.4
18:0	7.4	5.6±1.0	6.9±1.2	6.1±1.1	5.3±1.6	6.3±1.1
18:1 n9+n7	94.7	83.2±11.0^{ab}	87.9±11.5^{ab}	92.1±7.7^{a}	80.2±11.1^{ab}	75.9±10.9^{b}
18:2 n6	50.1	35.1±8.5	40.1±4.8	37.5±8.5	36.7±6.2	41.0±9.7
18:3 n3	11.4	4.6±1.1	6.1±1.8	4.3±1.9	4.0±1.0	5.3±1.3
18:4 n3	10.4	7.0±0.8^{b}	8.5±1.0^{a}	8.4±0.7^{a}	7.8±1.3^{ab}	7.9±1.0^{ab}
20:4 n6 (ARA)	6.1	5.9±1.7	6.2±1.5	7.0±1.7	6.3±1.3	6.8±1.1
20:3 n3	0.8	1.2±1.0	1.2±0.4	1.5±0.8	1.6±.05	1.9±0.5
20: n3 (EPA)	6.7	4.3±1.8^{d}	7.0±1.2^{c}	8.2±1.3^{bc}	10.4±3.2^{b}	14.1±2.7^{a}
21:5 n3	-	1.0±0.3	0.8±0.2	0.8±0.2	0.9±0.2	1.1±0.3
22:4 n6	-	0.7±0.4^{b}	1.4±0.4^{a}	1.4±0.3^{a}	1.4±0.4^{a}	1.6±0.4^{a}
22:5 n3	-	1.2±0.5^{b}	1.6±0.4^{ab}	1.5±0.5^{ab}	1.7±0.5^{ab}	2.0±0.5^{a}
22:6 n3 (DHA)	9.9	3.8±1.8^{c}	7.5±1.6^{b}	10.0±1.6^{b}	11.0±4.3^{b}	16.1±4.1^{a}
∑ SFA	72.2	50.3±4.9^{b}	61.6±10.1^{a}	58.0±7.6^{ab}	57.0±6.2^{ab}	54.6±8.9^{b}
∑ MUFA	147.4	125.8±11.0^{a}	135.8±18.8^{a}	134.1±14.5^{a}	124.5±13.7^{a}	105.2±15.6^{b}
∑PUFA(C≥18:2n)	98.9	65.4±7.2^{b}	79.2±14.4^{ab}	80.0±11.6^{ab}	79.4±11.3^{ab}	91.4±11.9^{a}
∑ n-3 PUFA	42.7	16.3±3.5^{d}	26.9±4.0^{c}	29.8±3.5^{bc}	33.2±5.9^{b}	41.3±3.0^{a}
∑ n-6 PUFA	56.2	48.7±7.3	53.4±7.4	49.4±8.1	47.9±6.8	56.9±0.1
∑ n-3/ ∑ n-6	0.8	0.3	0.5	0.6	0.7	0.8
∑HUFA(C≥20:3n)	27.0	21.5±5.2^{c}	27.0±4.7^{c}	33.8±5.9^{b}	37.8±6.4^{ab}	41.7±3.0^{a}
DHA/EPA	1.5	0.9	1.1	1.2	1.1	1.1

Data were presented as mean ± standard deviation (n=10). Values in the same column that do not share the same superscript are statistically significantly different (P<0.05)

Table 5. Fatty acid composition of ovary of *Eriocheir sinensis* broodstock at the start of the experiment and after being fed the experimental diets containing different HUFA levels for 3 months

Fatty acids (mg g^{-1} dw)	Initial	Diet 1 (0.14%)	Diet 2 (0.65%)	Diet 3 (1.15%)	Diet 4 (1.64%)	Diet 5 (2.07%)
16:0	26.3	23.2±3.0	22.6±2.3	22.3±1.7	22.9±2.7	22.1±1.6
16:1 n7	29.5	22.9±3.5^{a}	24.5±3.7^{a}	21.9±4.9^{ab}	21.8±2.9^{ab}	18.0±3.1^{b}
17:1 n7	-	1.0±0.3	1.1±0.1	1.2±0.3	1.2±0.2	1.0±0.1
18:0	5.8	4.9±0.7	4.9±0.4	4.7±0.6	4.7±0.8	5.1±0.6
18:1 n9+n7	55.2	50.5±5.5	49.0±3.9	48.7±2.3	47.0±5.2	45.0±5.9
18:2 n6	24.0	24.3±4.5	22.8±3.8	20.9±4.0	20.4±2.4	21.4±2.4
18:3 n3	6.7	5.2±1.9^{a}	4.6±1.6^{ab}	3.5±0.6^{ab}	3.5±0.6^{ab}	4.2±1.6^{b}
18:4 n3	4.6	2.9±0.7	2.7±0.5	3.0±0.7	3.0±0.5	2.8±0.3
20:4 n6 (ARA)	7.9	8.9±1.8^{a}	7.4±0.9^{ab}	7.6±1.8^{ab}	7.3±0.7^{ab}	7.1±1.1^{b}
20:3 n3	0.6	0.6±0.3	0.6±0.1	0.9±0.4	0.8±0.2	0.9±0.2
20:5 n3 (EPA)	9.3	13.0±3.4^{b}	14.6±2.7^{b}	18.5±2.1^{a}	19.4±2.0^{a}	20.6±2.7^{a}
21:5 n3	-	0.5±0.1	0.5±0.1	0.5±0.2	0.5±0.1	0.6±0.3
22:4 n6	-	0.6±0.2	0.6±0.4	0.9±0.3	0.8±0.2	0.5±0.2
22:5 n3	-	1.5±0.6	1.2±0.4	1.4±0.4	1.4±0.1	1.3±0.2
22:6 n3 (DHA)	10.6	10.1±2.6^{c}	11.8±1.8^{bc}	13.8±1.9^{ab}	13.8±1.5^{ab}	15.1±1.0^{a}
∑ SFA	32.1	28.1±3.5	26.9±3.0	26.6±1.6	27.6±3.4	27.3±1.9
∑ MUFA	84.7	74.4±7.2^{a}	74.5±6.2^{a}	71.8±4.7^{ab}	70.0±5.7^{ab}	64.1±8.1^{ab}
∑PUFA (C≥18:2n)	63.7	70.4±9.2	69.3±5.3	71.8±6.1	68.1±8.3	74.4±5.9
∑ n-3 PUFA	31.8	28.3±6.3^{b}	32.1±3.3^{b}	37.7±4.4^{a}	38.2±2.7^{a}	41.1±3.6^{a}
∑ n-6 PUFA	31.9	38.1±5.1^{a}	35.3±3.7^{ab}	32.5±3.1^{b}	31.6±2.0^{b}	33.1±3.6^{b}
∑ n-3/ ∑ n-6	1.0	0.7	0.9	1.2	1.2	1.3
∑HUFA(C≥20:3n)	28.4	35.8±7.2^{b}	39.6±4.0^{ab}	44.5±6.3^{a}	43.8±3.7^{a}	46.0±4.5^{a}
DHA/EPA	1.2	0.8	0.8	0.7	0.7	0.7

Data were presented as mean ± standard deviation (n=10). Values in the same column that do not share the same superscript are statistically significantly different (P<0.05)

Table 6. Fatty acid composition of muscles of *Eriocheir sinensis* broodstock fed the experimental diets containing different HUFA levels for 3 months

Fatty acids (mg g^{-1} dw)	Diet 1 (0.14%)	Diet 2 (0.65%)	Diet 3 (1.15%)	Diet 4 (1.64%)	Diet 5 (2.07%)
16:0	2.9±0.4^{abc}	2.9±0.5^{bc}	3.7±0.8^{ab}	2.7±0.4^{c}	4.0±0.5^{a}
16:1 n7	2.3±o.4^{a}	1.3±0.6^{b}	1.2±0.3^{b}	1.4±0.2^{b}	-
18:0	1.1±0.1	1.1±0.2	1.3±0.1	1.0±0.1	1.1±0.2
18:1 n9+1n7	8.0±1.0^{a}	6.7±1.1^{bc}	7.5±0.6^{ab}	5.9±0.6^{c}	6.1±1.0^{c}
18:2 n6	3.6±0.3^{a}	3.1±0.8^{a}	2.9±0.3^{a}	2.2±0.4^{b}	2.2±0.4^{b}
18:3 n3	-	-	-	-	-
20:4 n6 (ARA)	2.5±0.4^{a}	2.2±0.6^{a}	2.1±0.4^{a}	1.6±0.2^{ab}	1.4±0.3^{ab}
20:5 n3 (EPA)	5.3±1.0	5.4±1.0	6.4±0.5	5.4±0.6	5.3±0.8
22:5 n3	0.2±0.1	0.2±0.1	0.2	0.2±0.1	0.2
22:6 n3 (DHA)	3.8±0.6^{ab}	3.6±0.5^{b}	4.2±0.3^{a}	3.4±0.3^{b}	3.6±0.6^{ab}
∑ SFA	3.8±0.6	4.0±0.6	5.0±0.9	3.7±0.5	4.5±1.7
∑ MUFA	10.2±1.8^{a}	7.7±1.7^{bc}	8.2±0.5^{b}	7.0±1.0^{bc}	6.3±0.9^{c}
∑PUFA (C≥18:2n)	15.7±1.9^{ab}	15.4±2.5^{abc}	16.9±0.7^{a}	13.6±1.3^{bc}	13.3±2.3^{c}
∑ n-3 PUFA	9.6±1.6	9.6±1.5	11.5±0.6	9.6±1.1	9.8±1.7
∑ n-6 PUFA	6.1±1.0^{a}	5.7±1.3^{a}	5.4±0.5^{a}	4.0±0.8^{b}	3.7±0.8
∑ n-3/ ∑ n-6	1.6	1.8	2.2	2.3	2.4
∑HUFA(C≥20:3n)	12.1±1.6^{ab}	11.6±2.0^{ab}	13.2±0.8^{a}	10.8±0.9^{b}	10.8±1.5^{b}
DHA/EPA	0.7	0.7	0.7	0.6	0.7

Data were presented as mean ± standard deviation (n=10). Values in the same column that do not share the same superscript are statistically significantly different (P<0.05)

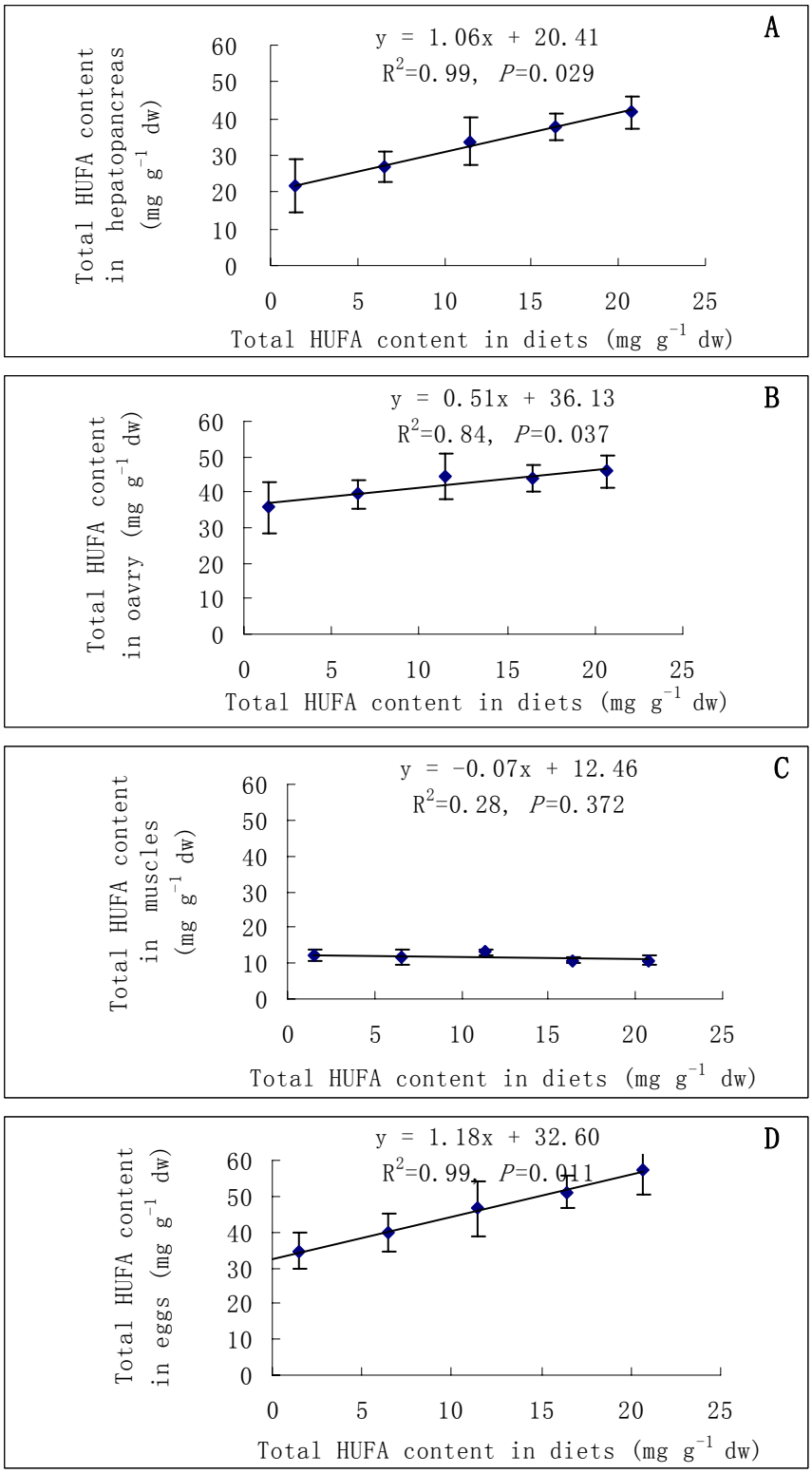

Fig. 2. Linear regression between the HUFA level in the diets and in the hepatopancreas (A), ovary (B), muscles (C) and eggs (D) of *Eriocheir sinensis* broodstock fed the experimental diets containing different HUFA levels for 3 (A, B and C) or 8 months (D). Data were presented as mean ± standard deviation (n=5)

Table 7. Fatty acid composition of eggs of *Eriocheir sinensis* broodstock fed the experimental diets containing different HUFA levels for 8 months

Fatty acids (mg g^{-1} dw)	Diet 1 (0.14%)	Diet 2 (0.65%)	Diet 3 (1.15%)	Diet 4 (1.64%)	Diet 5 (2.07%)
16:0	22.5±2.4	22.3±2.1	23.5±1.9	22.4±3.2	25.6±4.1
16:1 n7	37.4±7.0^{a}	30.5±4.1^{ab}	30.9±2.5^{ab}	28.2±7.1^{b}	33.0±7.0^{ab}
17:1 n7	1.1±0.4	1.1±0.3	0.9±0.2	0.8±0.2	1.2±0.3
18:0	3.0±0.2	3.7±0.2	3.2±0.4	3.4±0.3	3.7±0.4
18:1 n9+1n7	65.8±7.1	62.2±5.6	65.1±8.8	60.3±4.9	61.3±6.9
18:2 n6	34.9±5.6^{a}	31.3±3.4^{ab}	29.2±4.1^{b}	26.2±2.6^{b}	27.2±3.6^{b}
18:3 n3	7.9±1.4^{a}	8.6±1.8^{a}	6.8±1.2^{ab}	4.7±2.2^{b}	6.7±2.3^{ab}
20:4 n6	7.7±1.8	8.2±2.0	8.0±1.6	7.5±1.2	9.1±1.4
20:5 n3	13.0±3.1^{c}	16.8±2.9^{c}	21.1±3.3^{b}	24.8±1.7^{ab}	26.8±4.5^{a}
22:5 n3	1.1±0.1	1.2±0.3	1.2±0.2	1.3±0.4	1.4±0.3
22:6 n3	10.6±2.9^{d}	12.1±2.0^{cd}	14.7±2.9^{bc}	16.4±2.2^{ab}	18.3±3.3^{a}
∑ SFA	22.5±2.4^{b}	24.0±3.3^{b}	24.7±2.3^{ab}	24.6±4.5^{ab}	28.9±4.4^{a}
∑ MUFA	103.9±13.2	93.8±8.8	96.9±10.1	89.1±10.1	95.4±21.5
∑PUFA(C≥18:2n)	75.5±5.2^{b}	80.2±5.3^{b}	82.6±8.5^{b}	82.8±6.3^{b}	91.1±5.7^{a}
∑ n-3 PUFA	24.9±4.2^{d}	31.0±4.9^{c}	39.9±5.0^{b}	45.0±2.7^{ab}	46.0±3.9^{a}
∑ n-6 PUFA	51.9±5.9^{a}	48.6±4.5^{ab}	44.6±4.4^{bc}	37.6±4.0^{d}	42.6±3.2^{cd}
∑ n-3/ ∑ n-6	0.5	0.6	0.9	1.2	1.1
∑HUFA(C≥20:3n)	34.7±5.0^{d}	39.7±5.3^{cd}	46.5±7.5^{bc}	51.2±4.7^{ab}	57.6±7.0^{a}
DHA/EPA	0.8	0.7	0.7	0.7	0.7

Data were presented as mean ± standard deviation (n=10). Values in the same column that do not share the same superscript are statistically significantly different (P<0.05)

3.4 Effect of dietary HUFA on broodstock reproductive performance

20 days after copulation, the spawning percentages were similar for all treatments and ranged around 85% (Table 8). Most of the spawning occurred in the period of 1-10 days post-copulation (Fig. 3). Although no statistical analysis could be performed on the spawning percentage, there was a clear trend that the females fed Diets 5 and 4 spawned earlier (45-50% of the females spawned within 2 days post-copulation) than those in the other treatments (only 30-37% females spawned within 2 days post-copulation).

The average total egg production per female ranged from 35.7×10^4 for the females fed Diet 5 to 38.9×10^4 for the females fed Diet 1, and the average fecundity (eggs per gram female) ranged from 3400 for the females fed Diet 5 to 3645 for the females fed diet 1. Egg diameters ranged from 345 to 357 μm for the different treatments. No statistical differences were detected for any of these parameters among the treatments (Table 8)

(P>0.05).

Table 8. Spawning percentage, total number of eggs per female, fecundity and egg diameter of *Eriocheir sinensis* broodstock fed the experimental diets containing different HUFA levels for 8 months

Treatment	Spawning percentage (%)	Total number of eggs per female ($\times 10^4$)	Fecundity (eggs per gram female, excluding egg weight)	Egg diameter (μm)
Diet 1 (0.14%)	87	38.9 ± 6.4	3645 ± 545	345 ± 14
Diet 2 (0.65%)	80	38.8 ± 8.7	3644 ± 557	356 ± 11
Diet 3 (1.15%)	82	36.3 ± 6.8	3457 ± 602	351 ± 13
Diet 4 (1.64%)	85	36.4 ± 8.0	3475 ± 576	357 ± 8
Diet 5 (2.07%)	80	35.7 ± 6.7	3400 ± 737	355 ± 6

Data were presented as mean ± standard deviation (n=10). Values in the same column have no statistically significantly difference (P >0.05)

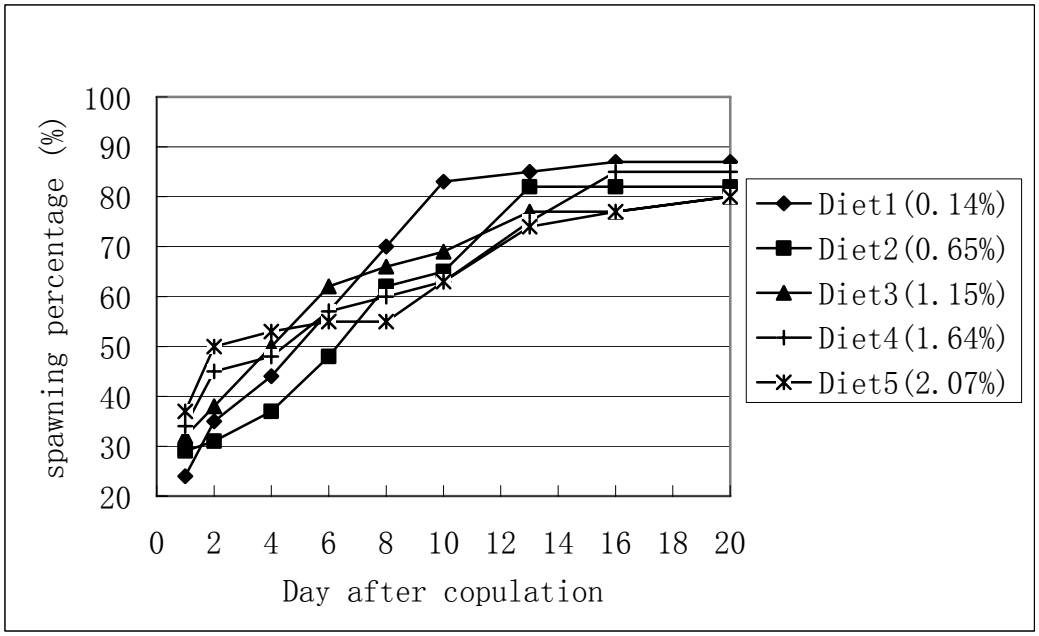

Fig. 3. Time course of spawning percentage of *Eriocheir sinensis* broodstock fed the experimental diets containing different HUFA levels for 8 months

4. Discussion

The results of the present study showed that, during the process of the first ovarian maturation of *Eriocheir sinensis*, both the HSI and the hepatic lipid content decrease significantly, while the GSI and the ovarian lipid content increase dramatically. This is in line with the observations by Cheng et al. (1998a) and Wen et al. (2001),

reconfirming that the hepatic lipid reserves are transferred to the developing ovaries during ovarian maturation of the female *Eriocheir sinensis*. On the other hand, no significant differences were observed in GSI of broodstock fed increasing dietary HUFA levels. This could be explained by the HUFA reserves in the hepatopancreas, which are utilized to maintain normal ovarian development and to obtain similar GSI. In this respect it was noted that the HSI and hepatic lipid content before starting the experiment were rather high (9% and 45.5% WW, respectively).

In this study, the dietary HUFA level significantly affected the fatty acid composition of the hepatopancreas, ovary and eggs, especially their EPA, DHA and total HUFA content, but no significant correlation could be found between the muscle HUFA level and the dietary HUFA content. It is known that in crustaceans, the hepatopancreas acts as storage organ for dietary lipids prior to its mobilization and incorporation in the tissues (Harrison, 1990; Wouters et al., 2001a). Therefore it is not surprising that, compared to the mature ovary and eggs, the fatty acid composition of the hepatopancreas was more markedly influenced by the diet. Similar results have been reported for other crustaceans by Alava et al. (1993), Xu et al. (1994), Cahu et al. (1999), Cavalli et al. (1999) and Wouters et al. (2001b). Furthermore our results showed that the ovary and eggs contained lower levels of MUFA and SFA, but higher level of HUFA than the hepatopancreas, which agrees with previous studies on *Pleoticus mulleri* (Jeckel et al., 1989), *Fenneropenaeus chinensis* (Ji and Xu, 1992) and *Eriocheir sinensis* (Cheng et al., 2000b; Wen et al., 2001). The difference in fatty acid composition between the hepatopancreas and mature ovary and eggs implicates the specific fatty acids requirement during the ovarian development of *Eriocheir sinensis*. Yet, SFA and MUFA may also have been catabolised for the production of energy while PUFA and HUFA were conserved (Cavalli et al., 1999).

In the present study, despite of a DHA/EPA ratio of 0.83-0.95 in the diets, the ovaries and eggs from all treatments generally had a lower DHA/EPA ratio (0.7-0.8) compared to the hepatopancreas (0.9-1.2). This may indicate that female *Eriocheir sinensis* require more EPA than DHA during ovary development (Wu, 2004; Wu et al., 2007d; 2007b).

Thus the excess DHA may be stored in the hepatopancreas, whilst more EPA was transported to the developing ovaries. The lower DHA/EPA ratio retained in the ovary and eggs of *Eriocheir sinensis* broodstock is in contrast with several study results on marine fish and crustaceans, where DHA was preferentially incorporated into egg lipids (Mourente and Odriozola, 1990; Harel et al., 1994). A possible explanation could be the catadromous life cycle of this species, in which the animals spend most of their life in freshwater and only move to brackish water to reproduce. Therefore, *Eriocheir sinensis* may not present the same fatty acid requirements as marine species (*in casu* a high DHA requirement), but rather present intermediate characteristics between freshwater and marine species, similarly to what was reported for the freshwater prawn *Macrobrachium rosenbergii* (Cavalli et al., 1999).

The present study showed that an increased dietary HUFA level had no significant effect on the total egg production and fecundity of the female *Eriocheir sinensis*. This is in agreement with the findings for penaeid shrimps, e.g. *Litopenaeus vannamei* (Cahu et al., 1994; Wouters et al., 2001c) and *Fenneropenaeus indicus* (Cahu et al., 1995), but in contrast with *Marsupenaeus japonicus* (Alava et al., 1993), *Fenneropenaeus chinensis* (Xu et al., 1994) and *Macrobrachium rosenbergii* (Cavalli et al., 1999), for which the dietary HUFA level had significant effects on GSI or fecundity. Such inconsistency may be explained by inter-specific differences in HUFA requirements during ovarian development (Cavalli et al., 1999; Wouters et al., 2001a). In this respect, our results also do not agree with a previous study on *Eriocheir sinensis* by Wen et al. (2002c), where positive effects of dietary HUFA (particularly EPA and DHA) on the fecundity and egg hatchability were observed. While the reproductive performance (spawning percentage, egg production per female and fecundity) of *Eriocheir sinensis* was very little influenced by the dietary HUFA level, it may be that egg or larval quality is more markedly affected. The effect of fatty acid composition of the broodstock diet on offspring quality has been demonstrated for other species (Xu et al., 1994; Cahu et al., 1995; Cavalli et al., 1999). This could however not be demonstrated here and needs further study. Another possible explanation why dietary HUFA levels did not affect reproductive performance might be the relatively large hepatic reserves before the start

of the first maturation as mentioned earlier. It is possible that these were sufficient to sustain normal development of the ovaries and successful spawning. In this respect it would be interesting to repeat the experiment with re-matured crab in which lipid reserves have been fully depleted after first spawning.

On the other hand, no significant difference could be found in ovarian EPA, DHA and HUFA content between Diet 3, 4 and 5 treatments, and egg EPA, DHA and HUFA content between Diet 4 and 5 treatments, meaning HUFA incorporation into the eggs reached a plateau at a dietary level of 1.64%. In this respect, previous studies have shown that *Eriocheir sinensis* eggs with higher HUFA or DHA content tended to have higher hatching rate or resulted in better larval quality (Wen et al., 2002c; Wu et al., 2004). Therefore it is reasonable to assume that in the current study, diets 4 and 5 resulted in better egg and larval quality than the other treatments. However, such an assumption warrants further research. Together with the observation that females fed diets with a higher HUFA level (Diets 4 and 5, 1.64 and 2.07%, respectively) spawned earlier than the other treatments, which may suggest that 1.64% HUFA in the formulated diet is required to meet the HUFA requirement of female *Eriocheir sinensis* during first maturation.

In summary, the quantitative requirement of dietary HUFA for *Eriocheir sinensis* broodstock remained undetected in this study. Females fed diets with a higher HUFA level however seemed to spawn earlier. Furthermore, eggs from females fed a high HUFA diet contained higher HUFA levels than the eggs from other treatments, which are believed to be crucial for the embryonic development of *Eriocheir sinensis*. Based on these spawning results and analytical data, an optimal dietary HUFA level of approximate 1.64% of total dietary dry weight may be hypothesized. The effect of dietary HUFA level on reproductive performance during re-maturation and the effect on offspring quality should be further investigated

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Chapter IV Section III

Effects of different diets on reproductive performance and HUFA composition of Chinese mitten crab *Eriocheir sinensis* broodstock during second spawning

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Abstract

Three natural diets (clams, sandworms and trash fish) and one artificial diet were fed to four groups of re-maturing *Eriocheir sinensis* broodstock for two months. Reproductive performance, larval quality and fatty acid composition of the eggs, gonads and hepatopancreas of the crabs were determined for second maturation process. The HUFA profile of the eggs, ovary and hepatopancreas of the crabs reflected that of the experimental diets, meaning there is an important transfer of nutrients from the exogenous feed to these tissues during second ovary maturation. Except the higher survival of starved zoea 1 larvae originating from crabs fed trash fish, and the bigger egg diameter from crabs fed clams, no significant differences between treatments were found for any of the other parameters (e.g. body weight, survival and fecundity).

Key words *Eriocheir sinensis*; broodstock nutrition; second spawning; larval quality

1. Introduction

Reproductive performance and larval quality are of primary importance for the success of the hatchery phase of mitten crab (Cheng, 2003). Although during the last two decades substantial improvements in hatchery techniques have resulted in large numbers of larvae being produced, poor and variable survival of the larvae is still one of the major bottlenecks in *Eriocheir sinensis* larval rearing (Wu, 2004).

Eriocheir sinensis is known to be able to fertilize more than one batch of eggs from a single mating (Zhang et al., 2002). Like other penaeid shrimp, broodstocks accumulate a lot of energy and nutrients in the hepatopancreas from the exogenous feed during their ovary development, and later on transfer these to the ovaries via haemolymph and thus the developing embryo until egg hatching (Harrison, 1990). Depending on the temperature, nutritional and ecological conditions, the interval between the first spawned egg hatching and second spawning ranges from 15 to 20 days. Nutritional requirements of broodstock can be investigated for both first and second spawning (Nan

et al., 2006; Yu et al., 2007). After the first spawning, nutrient and energy levels in the spent brood crabs are depleted and need to be replenished during this short period. Generally a lower fecundity and low larval quality has been observed for the second spawning. Therefore it seems more interesting to investigate the effect of diet on reproductive performance and larval quality during the second spawning process of *Eriocheir sinensis* broodstock.

2. Materials and Methods

2.1 Broodstock source

The experiment was carried out at the Tianjin Modern Fishery Technology and Engineering Center, Tianjin, China during the period of February to May, 2004. The mature female and male crabs were selected from a farm in Chongming, Shanghai and were shipped by airplane to the hatchery. The average body weight was 68.5 g for female and 116 g for male.

2.2 Experimental design

2.2.1 First spawning

After being stocked separately for one week to acclimate to the indoor captive conditions, male and female crabs (in ratio of 1:3) were placed in the same concrete tank containing 20 g L^{-1} diluted seawater at 15 °C. After 6 days, 150 berried females were selected and randomly distributed into 5 experimental tanks. Another 30 crabs were kept separately in order to replace crabs that died due to manipulation at early stage. The average body weight of selected spawn crabs was 71.3 g (ranging from 65 g to 78 g), and the average fecundity of spawn was 2578 eggs g^{-1} BW (body weight).

Fiber-glass tanks, separated into 4 units with PVC plates, were used as experimental tanks. Each unit had a surface of 2 m². Water depth was kept at 40 cm. Three units within the same tank were used as three replicates for each treatment, each replicate contained 10 crabs. The tanks were covered with a black plastic sheet and three pieces of 1-m PVC tube were placed in each unit to provide shelter. Water parameters were

monitored daily. Salt concentration was kept at 20 g L^{-1}, pH 8.2-8.3, DO 8.4-8.9 mg L^{-1}. Faeces and uneaten feed were removed daily by siphoning. Depending on ammonia and nitrite levels (less than 1 mg L^{-1} and 0.2 mg L^{-1}, respectively) water was changed every two or three days. The water temperature varied from 15°C to 17°C.

Three natural diets and one moist artificial diet were fed to the crabs: clams (*Sinonovacula constrica*, treatment TC), sandworm (*Nereis japonicus*, treatment TS), trash fish (*Chaeturichthys stigmatia*，treatment TT) and an artificial diet (treatment TA). Daily feeding ration was about 10 % of total body weight for natural diets and 1-3% for artificial diet. Feeding was done at 16 hrs. During the first week, dead crabs were replaced by crabs from the separate stock tank in order to compensate mortality due to manipulation.

2.2.2 Second spawning

In the beginning of April, the first spawn crabs hatched eggs at water temperature of 16-17°C. From then onwards spent crabs were checked every day for second spawning. The second spawning percentage was determined as follows:

a = b/c× 100%, where

a = second spawning percentage, b = number of second spawning crabs, c = number of survivals after the first spawn hatched.

Two berried crabs were sampled from each replicate: (6±2) days after spawning, crabs were weighed (BW) after removing outer water and then the total egg mass, gonad and hepatopancreas were removed using tweezers, placed onto blotting paper and weighed to the nearest 0.01g. Eggs, gonad and hepatopancreas were collected and kept at -20 °C for fatty acid analysis. An egg sample of about 0.010g was weighed on a digital balance (Mettler AE200) to the nearest 0.0001g and was separated gently using a needle. The number of eggs in the sample was counted under a binocular microscope (Nikon SMZ645). Total egg mass was then calculated by extrapolating the number of eggs in the known mass to the total egg mass. Egg diameter of 100 eggs from each crab was

measured using a microscope at 10×10 magnification (Nikon YS100). Fecundity was calculated as follows:

Fecundity = number of eggs /BW of crab (g)

The remaining crabs were placed into individual hatching tanks for larval quality test.

2.2.3 Larval quality test

After hatching, 100 zoea 1 (Z1) larvae were taken from each tank, put into beakers containing 500 mL diluted seawater (20 g L^{-1}) with gentle aeration. The larvae were subsequently starved until they died. Water temperature ranged between 19-20 °C. The survival was determined every day when water was renewed. Three replicates were conducted from one individual crab and three crabs from each treatment were tested.

2.2.4 Analysis of fatty acid, protein and crude fat

Fatty acid composition of tissues was determined by a direct transmethylation method according to a modified procedure of Lepage and Roy (1984). The resulting fatty acid methyl ester (FAME) were separated and identified on a HP-5890A gas chromatograph with capillary column SPTM –2330 (Supelco, INC). Identification was based on a standard reference mixture (Nu-Chek-Prep, USA). Protein content was determined by the Kjeldahl method and crude fat was analyzed by the Soxhlet method.

2.3 Statistical analysis

Data were presented as mean ± standard deviation and were subjected to statistical analysis using the software SPSS. Statistical differences among treatments were determined using one-way ANOVA. Tukey's multiple range test was applied to detect significant differences between means (P<0.05). Percentage data were arc-sin transformed prior to analysis. The linear regression correlations between the EPA, DHA content in the diets and in the hepatopancreas, ovaries and eggs of bloodstock were analyzed using General Regression Models. P<0.05 was regarded statistically significantly different or significant positive correlation.

3. Results

3.1 Feed composition

Among the experimental diets, trash fish contained the highest protein level (81.3%); whereas artificial diets contained the lowest (34.93%) (Table 1). Total lipid level in the artificial diet was the highest (12.23%), the others ranged from 7% to 9%. Also the fatty acid composition of the experimental diets varied considerably. The EPA content ranged from 3.95 mg g^{-1} dw in trash fish to 7.94 mg g^{-1} dw in sandworms; the DHA content ranged from 1.04 mg g^{-1} dw in sandworms to 9.70 mg g^{-1} dw in the artificial diet; the DHA/EPA ratio ranged from 0.13 in trash fish to 1.47 in the artificial diet.

Table 1. Proximate chemical and fatty acid composition of the experimental diets

	Clam	Trash fish	Sandworm	Artificial diet
Moisture (%)	85.27	78.54	86.42	30.34
Protein (% dw)	56.55	81.32	53.39	34.93
Crude fat (% dw)	7.78	7.52	9.02	12.23
Fatty acids (mg g^{-1} dw)				
18:2 n6	0.52	0.58	0.41	5.79
18:3 n3	1.19	0.11	0.34	1.67
18:4 n3	4.12	0.37	1.36	1.61
20:4 n6 (ARA)	1.69	1.63	0.75	2.45
20:5 n3 (EPA)	4.52	3.95	7.94	6.61
22:6 n3 (DHA)	3.91	4.13	1.04	9.70
DHA/EPA	0.87	1.05	0.13	1.47
Total HUFA*	10.12	9.71	10.61	18.75

* Total HUFA = ARA+EPA+DHA

3.2 Reproductive performance

There were no significant differences among treatments on crab body weight (Table 2). Although TS and TT resulted in higher fecundities than TA and TC, there was no statistically significant difference among treatments due to the high variation within treatments. Average egg diameter in TC was significantly higher than in TT and TA, whereas TS had intermediate values. Although TT gave a higher survival at 12 and 20 days after the first spawn hatched, there were no statistically significant differences in survival among the treatments. By the time the second spawn hatched, the survival in all treatment had dropped to 2%. 100 % of the crabs had spawned a second time 20 days after the first spawn.

Table 2. Average body weight (BW), fecundity, egg diameter and survival of second spawning *Eriocheir sinensis* broodstock fed different experimental diets

Treatment	Body weight (g)	Fecundity (eggs g^{-1} BW)	Egg diameter (mm)	Survival % (after first spawn hatched)		
				12 day	20 day	30 day
TC	74.79 ± 2.99	2203 ± 350	4.63 ± 0.12 [a]	78±18	32±8	2±0
TS	68.70 ± 3.55	2952 ± 413	4.35 ± 0.06[ab]	68±16	34±26	2±0
TT	70.72 ± 3.72	2929 ± 275	4.20 ± 0.04 [b]	84±6	48±27	2±0
TA	72.47± 1.87	2435 ± 427	4.21 ± 0.13 [b]	57±7	41±27	2±0

Data are shown as mean ± standard deviation (n=6). Values in the same column showing a different superscript are significantly different (P<0.05)
TC — *Eriocheir sinensis* broodstock fed with clams (*Sinonovacula constricta*)
TS — *Eriocheir sinensis* broodstock fed with sandworm (*Nereis japonicus*)
TT — *Eriocheir sinensis* broodstock fed with trash fish (*Chaeturichthys stigmatias)*
TA — *Eriocheir sinensis* broodstock fed with artificial diet

3.3 Larval quality

The survival of starved Z1 larvae is shown in Fig.1. The starved Z1 larvae were not able to develop into the Z2 stage, no matter how long they survived. Treatment TT gave the best quality larvae. In this treatment 50 % of the starved larvae could survive for 7 days after hatching (DAH7); whereas only a small percentage of the larvae from TC and TS could survive until DAH7 and larvae of treatment TA could only survive to DAH5.

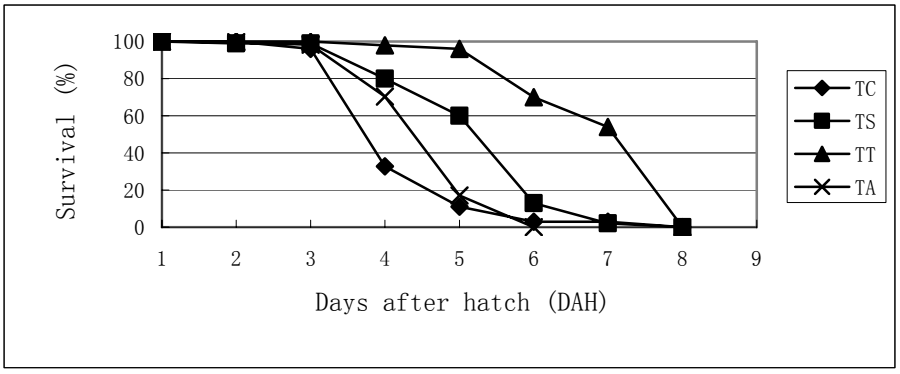

Fig. 1. Survival rate of starved zoea 1 larvae of *Eriocheir sinensis* obtained from second spawning broodstock fed different experimental diets. Refer to Table 2 for treatment description

3.4 Fatty acid composition of gonads, hepatopancreas and eggs

The fatty acid composition, and more specifically the DHA and the EPA contents in hepatopancreas, gonads and eggs reflected the composition of the diets (Table 3). Among treatments, eggs, gonad and hepatopancreas of crabs in TS had the highest EPA

content (18.95, 16.26 and 8.60 mg g^{-1} dw, respectively); whereas crabs in TT gave the lowest EPA value (11.60, 10.51 and 6.07 mg g^{-1}dw, respectively). On the other hand, TS resulted in the lowest DHA values in eggs, gonad and hepatopancreas (3.08, 2.60 and 0.81 mg g^{-1} dw, respectively), whereas TA resulted in the highest DHA values (12.82, 6.75 and 11.37 mg g^{-1} dw, respectively). Overall DHA content in both gonad and egg were less than EPA content in any treatment. In order to identify the correlation between dietary fatty acid composition and tissue fatty acid composition, linear regression analyses of tissue DHA and EPA content against dietary EPA and DHA contents were performed and shown in Fig. 2. The result showed that EPA and DHA content in eggs, and DHA content in hepatopancreas had significantly positive correlation with the dietary EPA and DHA contents (P<0.05); whilst dietary EPA and DHA had no significant influence on EPA content in hepatopancreas and gonad, and DHA content in gonad (P>0.05) .

Table 3. Average EPA and DHA content in eggs, gonads and hepatopancreas of second spawning *Eriocheir sinensis* broodstock fed different experimental diets

Fatty acids (mg g^{-1} dw)	Treatment	Eggs	Gonads	Hepatopancreas
EPA	TC	13.66 ± 2.58	10.93 ± 2.43	6.96 ± 1.53
	TS	18.95 ± 1.96	16.26 ± 1.78	8.60 ± 0.73
	TT	11.60 ± 2.39	10.51 ± 0.94	6.07 ± 0.71
	TA	15.83 ± 2.70	9.95 ± 2.38	9.64 ± 0.85
DHA	TC	8.90 ± 1.84	5.75 ± 1.45	2.95 ± 1.37
	TS	3.08 ± 0.55	2.60 ± 0.64	0.81 ± 0.21
	TT	7.52 ± 2.17	5.24 ± 0.97	4.83 ± 0.94
	TA	12.82 ± 2.40	6.75 ± 1.96	11.37 ± 0.81

Data are shown as mean ± standard deviation (n=6). Refer to Table 2 for treatment description

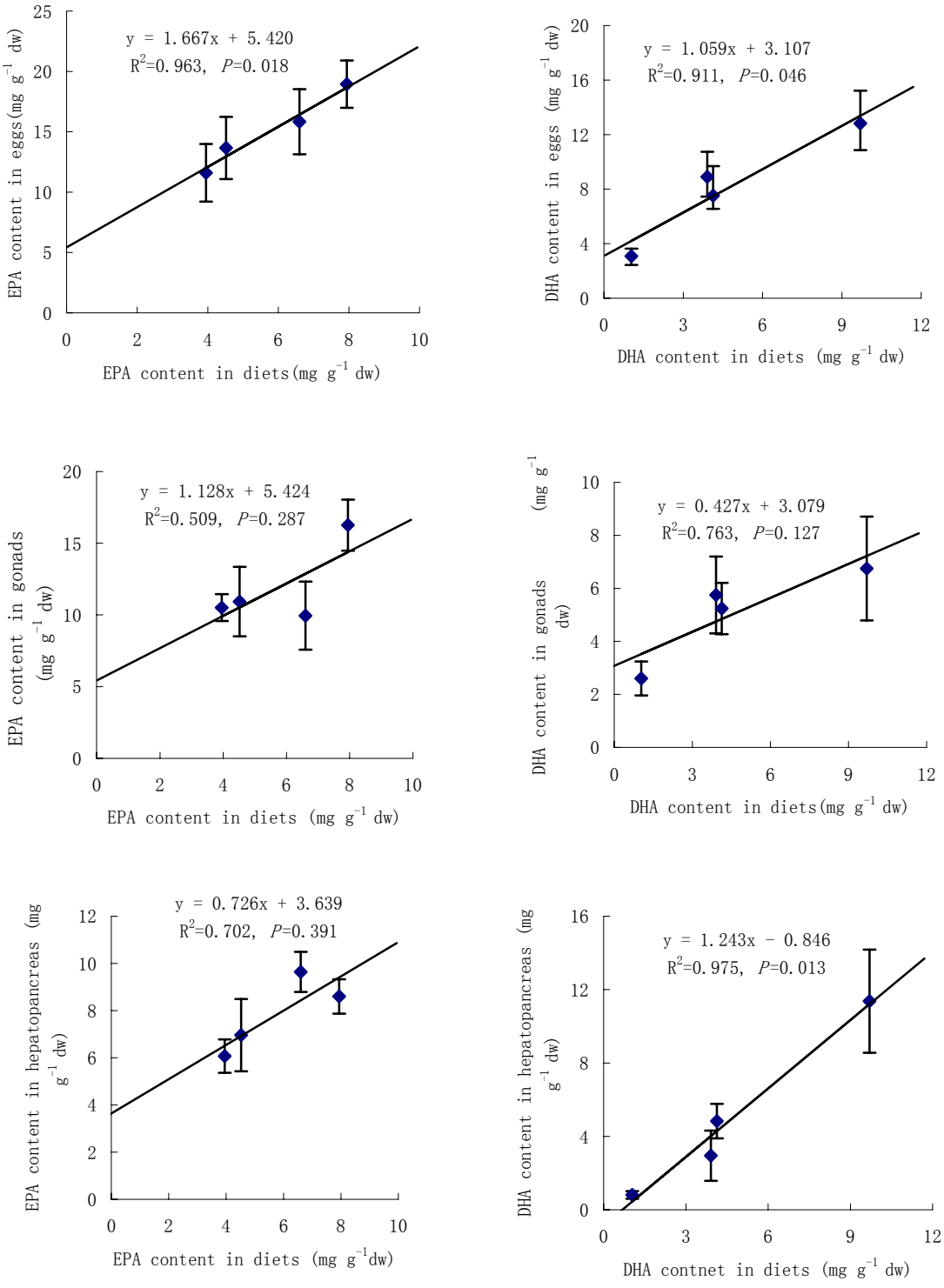

Fig. 2. Linear regression analysis of relationships between EPA, DHA content of diets and that in eggs, gonads and hepatopancreas of second spawning *Eriocheir sinensis* broodstock fed different experimental diets. Data were presented as mean ± standard deviation (n=6). Refer to Table 2 for treatment description

4. Discussion

The HUFA profile of the eggs, gonad and hepatopancreas of re-matured *Eriocheir sinensis* broodstock very well reflected that of the experimental diets (Fig. 2). This shows that, despite the large amounts of nutrients accumulated during the first ovarian maturation process, after first spawning, crabs need to take up a lot of energy and nutrients from exogenous diets to replenish its reserves, and transfer them via the hepatopancreas to the ovaries for secondary maturation. In this respect, the results from this study are in agreement with studies in other crustaceans, e.g. *Penaeus monodon* (Millamena, 1989), Fenneropenaeus *chinensis* (Xu et al., 1994), *Litopenaeus vannamei* (Cahu et al., 1994), *Macrobrachium rosenbergii* (Cavalli et al., 1999) and *Scylla paramamosain* (Djunaidah et al., 2003).

Furthermore the HUFA profile of these tissues probably is not merely influenced by the dietary HUFA level, but probably points out a specific requirement for these fatty acids (Castell, 1981; Xu et al., 1994). In this respect it was noted that the DHA and EPA content increased from the hepatopancreas, over gonad, to eggs, whereas the hepatopancreas had levels close to those of the diets, moreover DHA contents in both gonad and hepatopancreas were less than the EPA contents in any treatment. It is well understood that the hepatopancreas of crustaceans acts as a storage organ for dietary lipids prior to its mobilization and incorporation in specific tissues (Harrison, 1990). Although crustaceans have a limited ability to elongate fatty acids and eventually synthesize a certain amount of fatty acids *de novo*, the major part of the accumulated lipids in gonads and eggs originate from the diets through selective absorption (Kanazawa et al., 1979). As the lipids in the ovaries and eggs contain a higher proportion of n-3 HUFA, particularly EPA and DHA than those of the hepatopancreas, it is believed that they also play a crucial role in mitten crab reproduction (Teshima and Kanazawa, 1983). Studies on broodstock rearing of mitten crab (Wen, et al, 2002) using compound diets showed a high correlation between n-3 HUFA content in the eggs and reproductive performance of the broodstock. Xu et al. (1994) suggested that EPA may play some specific role in the ovary development process relating to fecundity, whereas

DHA may play some other role in early embryogenesis which related to egg hatchability of larval *Penaeus chinensis*. Our results showed that both EPA and DHA are important to ovary development and reproduction of mitten crab, of which EPA seems more crucial. However, no clear correlation could be obtained in this study.

It should however be noted that the different diets tested in this study, not only differed in HUFA content, but had a completely different gross (proteins, total lipids, etc.) as well as micro-nutrient (vitamins, minerals, sterols, phospholipids, carotenoids, etc.) composition. Therefore it is difficult to draw any definite conclusions. Moreover the reproductive performance of second spawning broodstock should also be related to that of first spawning. Unfortunately we did not record much data on the first spawning.

In all treatments mortality after second spawning was very high, especially upon hatching of the second spawn. This phenomenon is often observed in hatcheries. After two spawns the crabs have consumed a lot of their energy and nutrient reserves, hence they are nearly exhausted. From a nutritional point of view, this study shows that essential nutrients, e.g. HUFA can be supplemented through artificial diets to further enhance *Eriocheir sinensis* broodstock performance.

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Chapter V

Larval nutrition

Chapter V Section I

Ingestion of *Artemia* nauplii by Chinese mitten crab *Eriocheir sinensis* zoea larvae

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Abstract

A series of ingestion trials were conducted to determine the ingestion rate of *Artemia* nauplii by *Eriocheir sinensis* zoea larvae with increasing densities of *Artemia* and with or without rotifers as a co-feed. At each zoeal stage, 10 groups of 10 larvae were reared individually in glass beakers and fed with increasing densities of newly-hatched *Artemia* nauplii (0.5, 2.5, 5, 10 and 20 ind mL^{-1}) with or without rotifers (15-25 ind mL^{-1}) as a co-feed. The average number of ingested *Artemia* over 24 h was measured. In addition, the average larval development rate (Larval Stage Index, LSI) over a longer period (time needed for the best treatment to reach 100% molt or metamorphosis to the next larval stage) was compared. The results showed that *Artemia* ingestion rate of *Eriocheir sinensis* larvae increased with increasing prey densities and larval development. The ingestion rate of *Artemia* had significant negative correlation with rotifer consumption for all zoeal stages. Rotifers as an alternative prey significantly affected intake of *Artemia* at early larval stages (Z1 and Z2) and promoted LSI at lower *Artemia* density. Further experiments are needed to clarify the effect of prey density on the survival and larval development when larvae are reared communally.

Keywords *Eriocheir sinensis* larvae; *Artemia* nauplii; rotifers; prey ingestion

1. Introduction

Prey density is one of the factors affecting larval feeding success for all aquaculture species, in particular the brachyuran species that have strong cannibalistic behavior at larval stage. In addition, prey size is likely to be critical in terms of the ability of brachyuran larvae to effectively capture and ingest prey (Sulkin and Epifanio, 1975; Harvey and Epifanio, 1997; Davis et al., 2005a). The influence of prey concentration on larval survival and development has been studied in several brachyuran species, such as *Scylla serrata* (Suprayudi et al., 2002; Rescoe et al., 2004) and *Ranina ranina* (Minagawa and Murano, 1993a). Other studies emphasized on prey ingestion rate during a single day at each instar (Baylon et al., 2004; Minagawa and Murano, 1993b).

Despite long history and success of mitten crab larviculture, feeding protocol currently used in hatchery, to a large extent, still rely on the farmer's experience, little information exists on feeding regime of *Eriocheir sinensis* larvae. In this paper, the influence of increasing *Artemia* density with or without rotifer co-feeding on daily *Artemia* ingestion rate and larval development rate of individual *Eriocheir sinensis* zoea larvae was examined. Such information should provide basic knowledge to further develop an optimal rotifer and *Artemia* feeding regime in larval rearing system, which is studied in another paper.

2. Materials and Methods

2.1 Larvae stock culture

In order to have larvae at different stages of development to be used in the ingestion trials, a stock culture was maintained. Eggs were obtained by natural spawning from a captive breeder. The broodstock maintenance and egg incubation were operated as described by Sui et al. (2007). Upon hatching, positively photo-tactic zoea 1 (Z1) larvae were selected and placed into a 200 L plastic tank. The stock density was approximate 200 Z1 L^{-1}. The rearing temperature was kept at 21-22 ºC. Z1 and Z2 larvae were fed with rotifers *ad libitum*; and from Z3 onwards, newly-hatched *Artemia* nauplii were supplied *ad libitum*. Except for Z1 larvae, which were obtained directly from the hatching tank, Z2, Z3, Z4 and Z5 larvae for the ingestion trials were obtained from this stock culture.

2.2 Ingestion trials

Five ingestion trials were set up separately with each of the five zoeal stages. The trials started on the day when the eggs hatched onto the Z1 or the larvae molted into a new zoeal stage (Z2, Z3, Z4 and Z5), and ended when in one of the treatments 100% of the larvae had molted or metamorphosed to the next zoeal stage. The number of prey ingested within 24 h at second rearing day was determined for each zoeal stage with increasing densities of *Artemia* (0.5, 2.5, 5, 10 and 20 ind mL^{-1}) and with or without rotifers (15-25 ind mL^{-1}) as a co-feed. Hence in total, 10 treatments were conducted at

each larval stage. Each treatment consisted of 10 individually reared larvae.

20- mL glass beakers were filled with 8 mL diluted seawater (20 g L^{-1}). After 3 h starvation, a single larva was gently pipetted into the beaker and fed the appropriate number of *Artemia* and rotifers according to the treatment. Water temperature was kept in the range of 21-22 ºC by standing the beakers in a temperature controlled shallow water bath. Photoperiod was provided at 12L:12D with white fluorescent tubes. After 24 h, larvae were gently transferred into new beakers containing the same density of rotifers and *Artemia*. The remaining prey in the beaker was fixed with Lugol's solution before being counted under a binocular (Nikon SMZ645). Replicates containing dead larvae were eliminated from the ingestion rate calculation, no matter how long they had survived. Since it was possible for rotifers to reproduce in the beakers within 24 h, five replicates per *Artemia* density but without crab larvae were set up as controls. In computing the number of rotifers ingested per larva, the mean density of rotifers after 24 h was used to correct the initial number of rotifers present in the beakers. Partially consumed *Artemia* were included in counting the prey number left in the beakers. In addition to prey ingestion, larval development rate was also monitored daily by identifying the stage of each larva in the beakers, and was presented as Larval Stage Index (LSI) by assigning it a value: Z1=1, Z2=2, etc. to megalopa M=6 (Millamena and Bangcaya, 2001).

2.3 Rotifers and Artemia nauplii

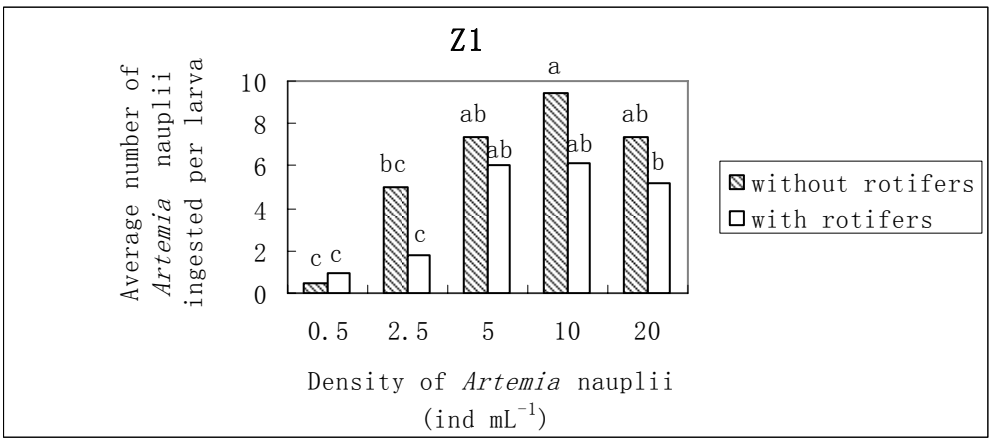
Rotifers (*Brachionus rotundiformis,* 45-180 μm in length) were mass cultured in a 100-L cylindrical PVC tank and fed with *Chlorella* sp.. *Artemia* cysts (strain from Bohai Bay, China) were hatched as described by Van Stappen (1996), and newly-hatched first instar nauplii (425-550 μm in length) were harvested after 18-20 h incubation. The density of rotifers in the stock was estimated by taking three samples at different locations. The number of *Artemia* nauplii were counted one by one using a glass pippet and transferred into glass beakers with the crab larvae.

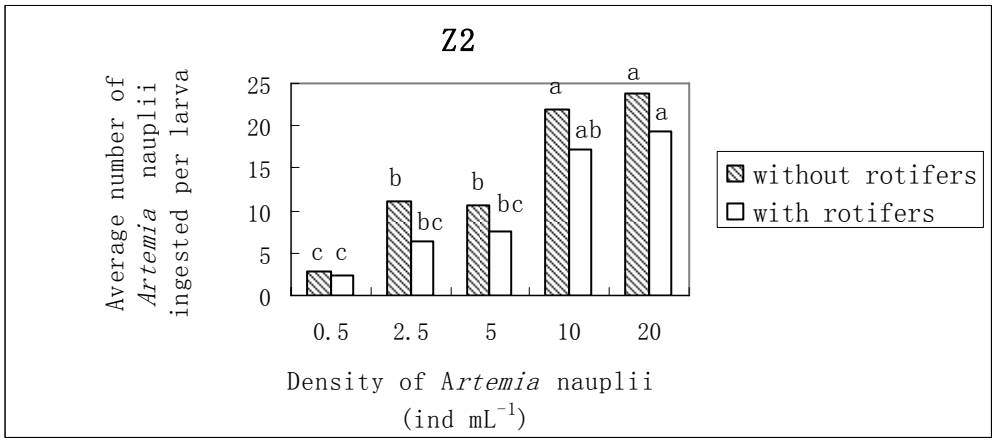
2.4 Statistical analysis

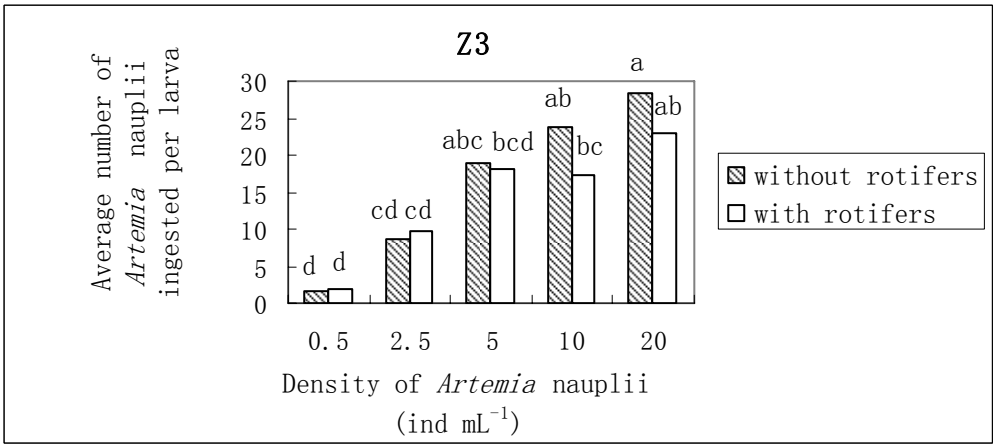
Data were presented as mean ± standard deviation. Data were subjected to statistical analysis using the software Statistica. Homogeneity of variance was tested with the Levene's test. Two-way ANOVA was used to determine if there were significant differences in the mean number of nauplii ingested at increasing densities of *Artemia* with or without rotifers co-feeding. Statistical significance of difference in LSI among treatments with or without rotifer co-feeding was determined separately using one-way ANOVA. Tukey's Unequal Number HSD of multiple range test was applied to detect significant differences between means. The linear regression correlations between the total number of *Artemia* and rotifer ingested by a single larva at each zoeal stage were analyzed using General Regression Models. $P<0.05$ was regarded statistically significantly different or significant linear correlation.

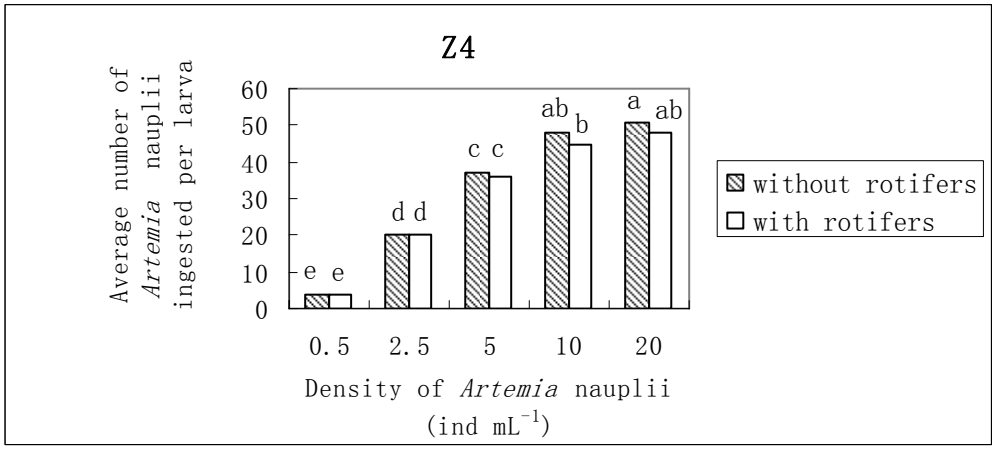
3. Results

There was a trend of increasing intake of *Artemia* with larval development (Fig. 1). Within the tested range, there was also a general tendency of increased consumption of *Artemia* with increasing *Artemia* densities in all zoeal stages The highest *Artemia* intake was obtained for the highest density (20 ind mL^{-1}) and the lowest ingestion was observed for the lowest density (0.5 ind mL^{-1}) in the Z2 to Z5 stage; whilst the highest ingestion rate was observed at a density of 5 ind mL^{-1}in Z1 stage. However, the statistical analysis showed no significant increase in *Artemia* consumption above 5, 10, 10, 10 and 20 ind mL^{-1} in the Z1, Z2, Z3, Z4 and Z5 stage, respectively.




Z2
Average number of Artemia nauplii ingested per larva
25
20
15
10
5
0
c c
b
bc
b
bc
a
ab
a
a
0.5
2.5
5
10
20
Density of Artemia nauplii (ind mL-1)
without rotifers
with rotifers


Z3
Average number of Artemia nauplii ingested per larva
30
25
20
15
10
5
0
d d
cd cd
abc bcd
ab
bc
a
ab
0.5
2.5
5
10
20
Density of Artemia nauplii (ind mL-1)
without rotifers
with rotifers


Z4
Average number of Artemia nauplii ingested per larva
60
50
40
30
20
10
0
e e
d d
c c
ab
b
a
ab
0.5
2.5
5
10
20
Density of Artemia nauplii (ind mL-1)
without rotifers
with rotifers

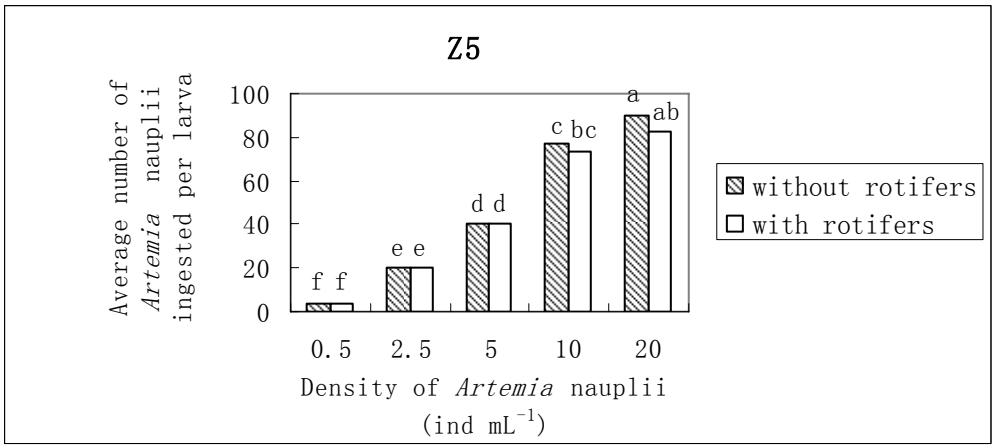
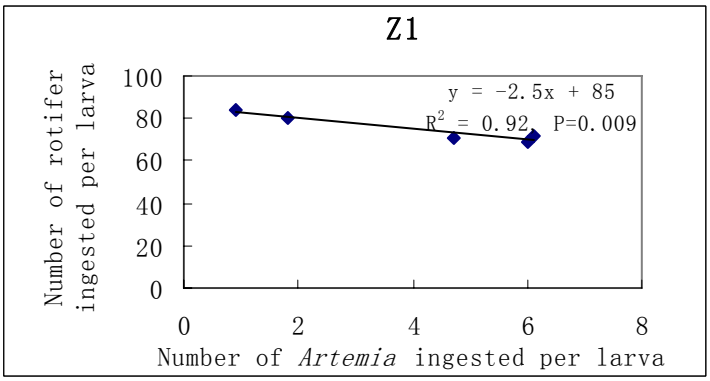


Fig. 1. Ingestion of *Artemia* nauplii by *Eriocheir sinensis* zoea larvae (Z1-Z5) with increasing *Artemia* density with or without rotifer co-feeding over 24 h period. Data were presented as mean ± standard deviation (n=10). Different letters present statistically significant difference among different *Artemia* densities with or without rotifer co-feeding (P<0.05)

The linear regression analysis revealed significantly negative correlations between the total number of *Artemia* and rotifer ingested by a single larva (P<0.05, n=5) (Fig. 2) for the treatments co-fed with rotifers for all larval stages. Meanwhile, the presence of rotifers as an alternative prey did significantly affect intake of *Artemia* at early larval stages (P=0.0009 for Z1, P=0.0064 for Z2); whereas no significant influence was observed in later zoeal stages (P=0.2786 for Z3, P=0.0769 for Z4 and P=0.0830 for Z5) at significance level of 0.05. Moreover, two-way ANOVA detected no significant interaction between rotifer feeding and *Artemia* density at any of the larval stages (P>0.05).



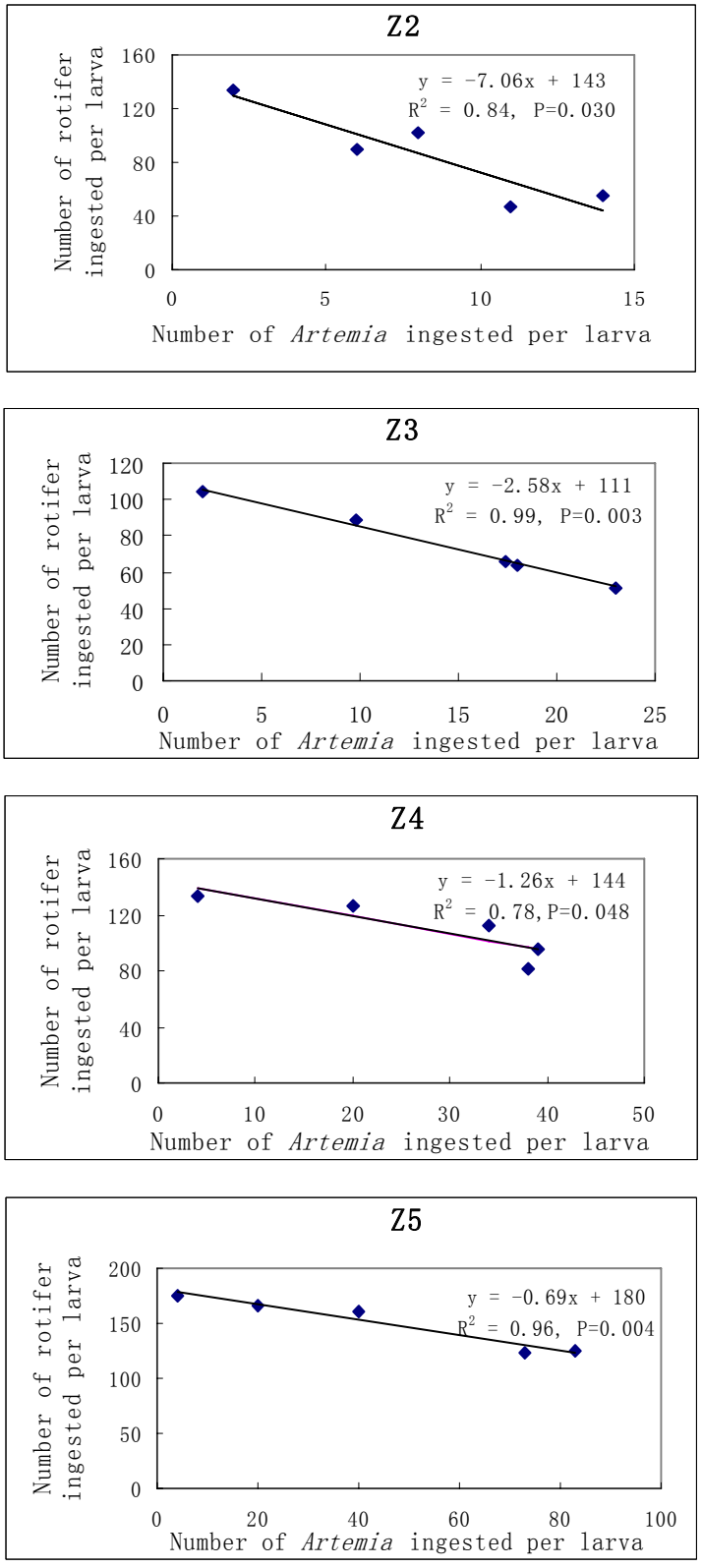


Fig.2. Linear regression correlation between the total number of *Artemia* and rotifer ingested by a single *Eriocheir sinensis* larva at each zoeal stage (Z1-Z5) (n=5)

The larval development (LSI) was also influenced by the *Artemia* density and the presence of rotifers (Table 1). When no rotifers were offered, *Artemia* densities of 0.5 ind mL^{-1} in the Z1, Z2 and Z3 stage, and *Artemia* densities of 0.5 and 2.5 ind mL^{-1} in the Z4 and Z5 stage significantly extended larval development ($P<0.05$). When rotifers were co-fed, the effect of *Artemia* density on LSI was not significant in the Z1, Z2 and Z3 stage, but *Artemia* density of 0.5 ind mL^{-1} resulted in significant low LSI in the Z4 and Z5 stage.

Table 1. Comparison of larval development rate (Larval Stage Index, LSI) of *Eriocheir sinensis* zoea larvae (Z1-Z5) fed *Artemia* nauplii with increasing *Artemia* density with or without rotifer co-feeding

Zoeal stage (rearing hour)	*Artemia* density (ind mL^{-1})	Larval Stage Index (LSI)	
		With rotifer	Without rotifer
Z1 (72h)	0.5	1.89 ± 0.33^{A}	1.00^{b}
	2.5	1.75 ± 0.46^{A}	1.22 ± 0.44^{ab}
	5	2.00^{A}	1.70 ± 0.48^{a}
	10	2.00^{A}	1.63 ± 0.52^{a}
	20	2.00^{A}	1.67 ± 0.50^{a}
Z2 (72h)	0.5	3.00^{A}	2.00^{b}
	2.5	3.00^{A}	2.67 ± 0.52^{a}
	5	2.71 ± 0.33^{A}	3.00^{a}
	10	2.86 ± 0.38^{A}	3.00^{a}
	20	2.83 ± 0.41^{A}	2.83 ± 0.41^{a}
Z3 (72h)	0.5	3.50 ± 0.53^{A}	3.00
	2.5	4.00^{A}	3.78 ± 0.44^{a}
	5	4.00^{A}	3.83 ± 0.41^{a}
	10	3.67 ± 0.51^{A}	4.00^{a}
	20	3.75 ± 0.46^{A}	4.00^{a}
Z4 (72h)	0.5	4.00^{B}	4.00^{b}
	2.5	4.60 ± 0.52^{A}	4.20 ± 0.42^{b}
	5	4.90 ± 0.33^{A}	4.80 ± 0.42^{a}
	10	4.80 ± 0.42^{A}	4.89 ± 0.33^{a}
	20	5.00^{A}	4.80 ± 0.42^{a}
Z5 (120h)	0.5	5.10 ± 0.32^{B}	5.00^{c}
	2.5	5.60 ± 0.52^{A}	5.40 ± 0.52^{b}
	5	5.78 ± 0.44^{A}	5.78 ± 0.44^{a}
	10	6.00^{A}	6.00^{a}
	20	6.00^{A}	5.90 ± 0.31^{a}

Data were presented as mean ± standard deviation (n=10). Different capital letters present

statistically significant difference among different *Artemia* densities with rotifer co-feeding ($P<0.05$); different small letters present statistically significant difference among different *Artemia* densities without rotifer co-feeding ($P<0.05$)

4. Discussion

The results from this study indicated that *Eriocheir sinensis* Z1 larvae are capable of consuming *Artemia* nauplii and that a single *Artemia* diet can sustain their survival and growth. Nevertheless, *Artemia* nauplii with missing body parts (mostly only head and appendages were eaten) were often observed at these stages, indicating that complete ingestion of *Artemia* might still be difficult and a smaller prey organism might be advantageous at early larval stages. Previous studies on brachyuran species stated that in early larval stages, rotifers should be provided; whereas from Z3 onwards, *Artemia* are preferred (Sulkin and Epifanio, 1975; Zeng and Li, 1992; Takeuchi et al., 1999; Davis et al., 2005a; 2005b). In addition, Baylon and Failaman (1997) reported that for *Scylla serrata* larvae, a combination of *Artemia* and rotifers resulted in a higher survival from Z1 to megalopa compared to only *Artemia* or rotifers. Our results confirm these recommendations. As rotifer co-feeding significantly affected *Artemia* consumption in the Z1 and Z2 stages, and resulted in a better larval development (LSI) without any effect of *Artemia* density, rotifer co-feeding at early *Eriocheir sinensis* larval stages should be recommended.

A general increase in ingestion with increasing density of *Artemia* was also observed in the current study, which is in parallel with the observation with the red frog crab *Ranina ranina* (Minagawa and Murano, 1993a; 1993b). This is likely due to increased chances of encounter between zoeal larvae and their prey. Crustacean larvae are generally passive feeders and do not pursue prey actively (Heasman and Fielder, 1983). They only feed if there is a chance of encounter with a food organism. However, in this study, excessive *Artemia* density did not significantly improve larval development rate as well, especially in the early larval stages. In practice, when added at too high densities, live food organisms in culture tanks compete with the larvae for space and available dissolved oxygen. Furthermore they contribute metabolites and bacterial load, which

can foul the water and cause visual confusion, stress and mortalities of the crab larvae (Baylon et al., 2004). On the other hand, food shortage usually results in cannibalism in crab larvae cultures (Takeuchi et al., 1999; Suprayudi et al., 2002). The single larvae rearing system and inconsecutive rearing protocol applied in the present study have ignored the above factors and no pronounced difference in survival rate was observed. Therefore the effect on survival over a long rearing period could not be investigated. Further studies are needed to investigate the optimal feeding regime in the communal larvae rearing system.

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Chapter V Section II

Effect of feeding scheme and prey density on survival and development of Chinese mitten crab *Eriocheir sinensis* zoea larvae

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Abstract

The effect of feeding scheme and prey density on survival and development of *Eriocheir sinensis* zoea larvae was studied in three experiments. Different combinations and densities of rotifers (*Brachionus rotundiformis*) and newly-hatched *Artemia* nauplii were fed to zoea larvae. Average survival at each stage, larval development (Larval Stage Index, LSI), duration of zoeal stage and individual megalopa dry weight were compared among treatments. This study revealed that, under the experimental conditions, rotifers should be replaced with *Artemia* between the Z3 and Z4 stage. The optimal rotifer feeding density for Z1 and Z2 was 15 and 20 mL^{-1}, respectively; while the optimal *Artemia* feeding density for Z3, Z4 and Z5 was 3, 5 and 8 mL^{-1}, respectively. Further trials in production scale are recommended.

Keywords *Eriocheir sinensis* larvae; feeding schemes; rotifer density; *Artemia* density

1. Introduction

Chinese mitten crab *Eriocheir sinensis* is one of the most popular aquaculture species in China. As a catadromus species, *Eriocheir sinensis* larvae hatch and develop in a marine environment, where they pass through five zoeal stages and one megalopa stage (Liang et al., 1974). The duration of larval development is usually 3-4 days for zoea 1 (Z1), 2-3 days for Z2-Z5, 4-7 days for megalopa depending on the rearing temperature, food availability and water salinity. Rotifers and *Artemia* are, for the time being, the most practical live food for mitten crab rearing, accounting for more than 30-40 % of the expenses entailed in a hatchery operation (Yu Xuequan, Tianjin Modern Fishery Technology and Engineering Center, personnel comm.).

Though a large hatchery output of mitten crab is achieved with a total megalopa yield of 521,893 kg (0.16-0.2 million megalopa per kg) in 2003 (China Fisheries Yearbook, 2004), the feeding regimes currently applied in mitten crab larval rearing are to a large extent based on visual observations and the experience of the farmer. This often results

in either underfeeding or food wastage and consequently inconsistent and low larval survival and growth. So far only a few studies dealing with feeding schemes for *Eriocheir sinensis* larvae have been published. Jiang et al. (2000) suggested that the optimal feeding ration is 60 rotifers mL^{-1} for Z1 and Z2, and *Artemia* should be offered to Z3 at a density of 10 mL^{-1}. Yin and Wang (2003) studied rotifer feeding rations with supplementation of microalgae and recommended rotifer densities of 20 and 30 rotifers mL^{-1} for Z2 and Z3, respectively. However, no comprehensive study to date has thoroughly investigated both the feeding scheme and optimal prey density.

The aim of the current study was to optimize the larval feeding scheme in terms of time for weaning the larvae from rotifers to *Artemia* and the feeding density of rotifers and *Artemia* at different larval stages in the larval rearing system. The information derived from this study should lead to improved larval feeding regimes and hence more sustainable and profitable hatchery production of *Eriocheir sinensis*.

2. Materials and Methods

2.1 Source of larvae

Three experiments were carried out at the Tianjin Modern Fishery Technology and Engineering Center on the northeast coast of China in 2005 and 2006. Eggs were obtained from captive *Eriocheir sinensis* broodstock. Broodstock management and egg incubation techniques were described by Sui et al. (2007). Upon hatching, Z1 larvae from a single breeder were kept communally and fed on rotifers *ad libitum*. In order to avoid high mortality at the beginning of the experiment, larvae were distributed into the experimental set-up and consequently the experiment started only one day after hatch (DAH1). On DAH1, only positively photo-tactic Z1 were selected and these were distributed randomly into the experimental larval rearing facilities.

2.2 Experimental design

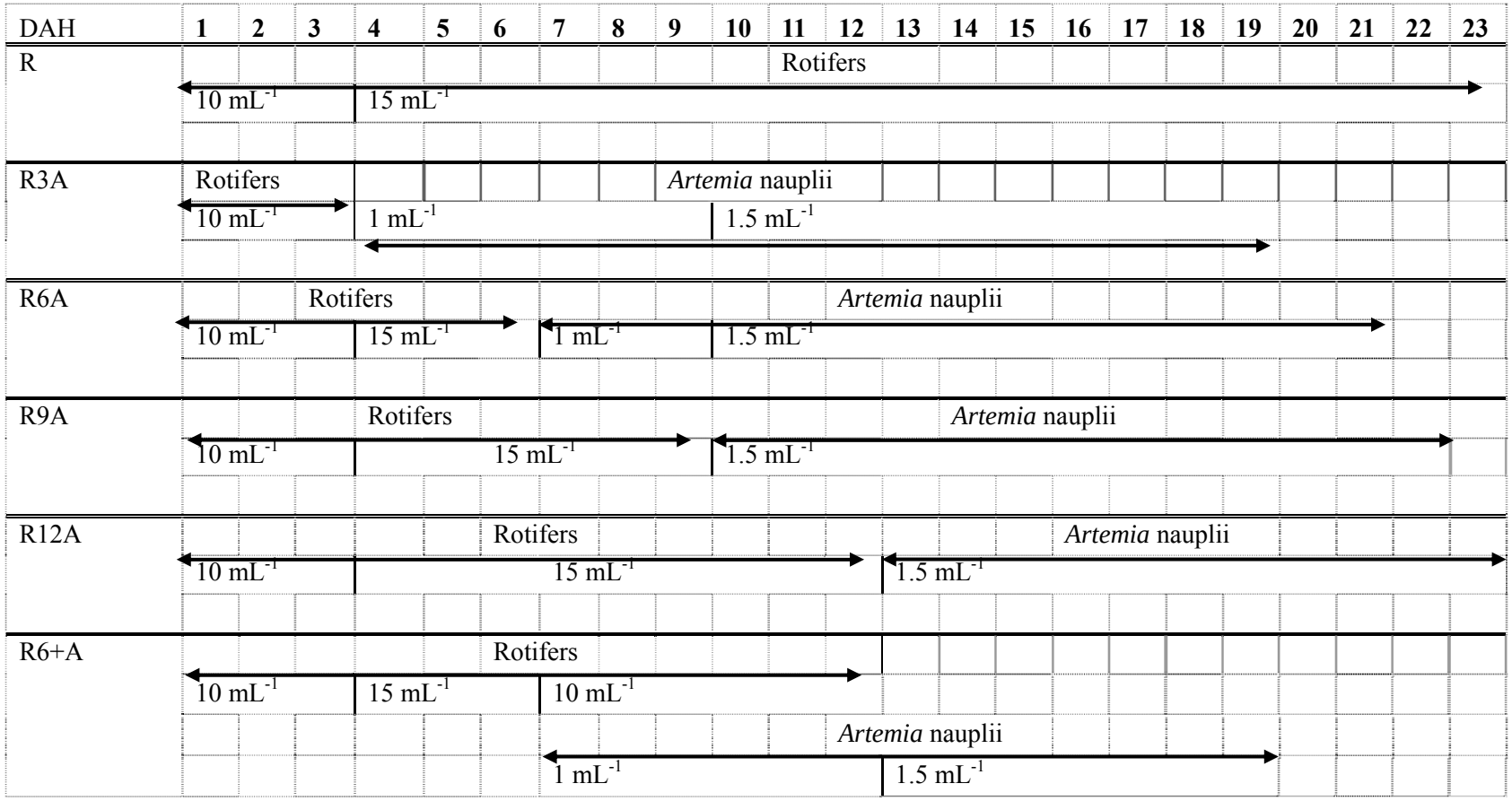
Experiment I was conducted to investigate the optimal time for weaning zoea larvae from rotifers to *Artemia*. Six different feeding schedules were applied (Table 1): Only

rotifers (R); Replacement of rotifers with *Artemia* at DAH3, when Z2 first appear (R3A); Replacement of rotifers with *Artemia* at DAH6, when Z3 first appear (R6A); Replacement of rotifers with *Artemia* at DAH9, when Z4 first appear (R9A); Replacement of rotifers with *Artemia* at DAH12, when Z5 first appear (R12A) and introduction of *Artemia* at DAH6 with rotifer co-feeding till DAH9 (R6+A). Feeding density of rotifers was 10 mL^{-1} for Z1, 15 mL^{-1} from Z2 onwards. The *Artemia* density was 1 mL^{-1} for Z2 and Z3 and 1.5 mL^{-1} for Z4 and Z5, respectively.

In Experiment II the optimal rotifer feeding density during the early zoeal stages was assessed. Seven different rotifer densities were fed to the Z1, Z2 and Z3 larvae (Table 2): 1/5/1 mL^{-1} (TR1); 5/10/5 mL^{-1} (TR2); 10/15/10 mL^{-1} (TR3); 15/20/15 mL^{-1} (TR4); 20/30/20 mL^{-1} (TR5); 30/40/30 mL^{-1} (TR6) and 40/50/40 mL^{-1} (TR7) for Z1/Z2/Z3, respectively. From the beginning of Z3 onwards, 0.5, 1.5 and 2.5 *Artemia* mL^{-1} were fed to the Z3, Z4 and Z5 stage, respectively in all treatments. Larvae were co-fed rotifers and *Artemia* at the Z3 stage.

In Experiment III the optimal *Artemia* feeding density during later zoeal stages was determined. Z1 and Z2 were communally reared in a stock tank and fed rotifers *ad libitum*. From the beginning of Z3 onwards, seven different *Artemia* densities were fed to the Z3, Z4 and Z5 larvae (Table 3): 0.5/0.7/1 mL^{-1} (TA1); 1.5/2.5/4 mL^{-1} (TA2); 3/5/8 mL^{-1} (TA3); 6/10/15 mL^{-1} (TA4); 10/15/20 mL^{-1} (TA5); 15/20/25 mL^{-1} (TA6); 20/25/30 mL^{-1} (TA7), respectively.

Table 1. Feeding schemes for *Eriocheir sinensis* larvae used in Experiment I



2.3 Larval rearing

Six replicates per treatment were performed using plastic beakers containing 1.5 L diluted seawater (20 g L^{-1}). The bottles were stocked with 150 Z1 in Experiment I, 200 Z1 in Experiment II and 150 Z3 in Experiment III. Water temperature was kept at 21-22ºC by the use of a heated water bath. pH ranged between 8.1-8.4. Gentle aeration was provided at Z1 and gradually increased at each stage, to become relatively strong at Z5 in order to prevent cannibalism. Dissolved oxygen was kept above 8 mg L^{-1}. A 12L:12D photoperiod was provided with white fluorescent tubes. Light intensity could not be measured, but was rather dim as only two 40 W lights were fixed at a height of 4-5 meters above the tanks. 100% water was renewed every other day when survival was determined. At that time, the content of the plastic beakers was gently poured into a bowl and the surviving larvae were counted while being pipetted into new beakers containing new rearing water. New beakers were prepared to the same temperature beforehand.

Rotifers (*Brachionus rotundiformis,* 45-180 µm in body length) were mass cultured in a 100-L cylindrical PVC tank and fed dry *Chlorella* sp. powder. Rearing temperature was 30°C and water salinity was 25 g L^{-1}. Rotifers were harvested over a 50µm screen and rinsed with clean seawater before use. *Artemia* cysts were hatched and newly-hatched first instar nauplii (425-550 µm in body length) were harvested as described by Van Stappen (1996). *Artemia* nauplii which could not immediately be fed to the larvae were kept at approximate 7 ºC to minimize metabolism and growth before feeding them to the larvae (Merchie, 1996). Feeding of the larvae was done three times per day (10 hrs, 16 hrs and 22hrs). Prior to the addition of new rotifers and *Artemia* to the beakers, the density of rotifers and *Artemia* in the stock and those remaining in the larval rearing beakers were estimated by analyzing three samples at different locations in the tanks and beakers.

Table 2. Feeding densities of rotifers and *Artemia* in Experiment II

Treatment	TR1	TR2	TR3	TR4	TR5	TR6	TR7	
Z1	1	5	10	15	20	30	40	(Rotifer. mL^{-1})
Z2	5	10	15	20	30	40	50	(Rotifer. mL^{-1})
Z3	1	5	10	15	20	30	40	(Rotifer. mL^{-1})
	0.5 (*Artemia*. mL^{-1})							
Z4	1.5 (*Artemia*. mL^{-1})							
Z5	2.5 (*Artemia*. mL^{-1})							

Table 3. Feeding densities of *Artemia* in Experiment III

Treatment	TA1	TA2	TA3	TA4	TA5	TA6	TA7	
Z3	0.5	1.5	3	6	10	15	20	(*Artemia*. mL^{-1})
Z4	0.7	2.5	5	10	15	20	25	(*Artemia*. mL^{-1})
Z5	1	4	8	15	20	25	30	(*Artemia*. mL^{-1})

2.4 Evaluation parameters

Survival percentages were calculated on the initial number of Z1 and Z3 larvae in Experiment I and II, and Experiment III, respectively. Survival was determined upon water renewal as described above. Although survival was checked only every other day, these days were chosen in such a way that the main molting events were covered. Survival data are expressed as survival at each zoeal stage, when more than 90% of the larvae reached that stage. In the metamorphosis period from Z5 to megalopa, megalopa were counted and then removed from the beakers as they appeared in order to prevent cannibalism on the remaining Z5 larvae. When all Z5 larvae had molted to megalopa, the experiment was terminated. 10-20 newly-molted megalopa were sampled at the same day from each replicate and the individual megalopa dry weight was determined (60 ºC, 24 h).

Larval development was monitored every other day and an average Larval Stage Index (LSI) was calculated as described by Millamena and Bangcaya (2001) by identifying the zoeal stage of each larva (n=6) and assigning it a value: Z1=1, Z2=2, etc. to megalopa M=6. The duration of zoeal stage was determined as the day when the LSI reached 5.90 (LSI≥5.90).

2.5 Statistical analysis

Data were presented as mean ± standard deviation and were subjected to statistical analysis using the software SPSS. Percentage data were arc-sin square root transformed prior to analysis. Homogeneity of variance was tested with the Levene's statistic. Statistical significance of differences among treatments was determined using one-way ANOVA. Tukey's multiple range test was applied to detect significant differences between means ($P<0.05$).

3 Results

3.1 Experiment I

Results of experiment I are presented in Table 4 and Table 5. There was no significant difference in survival among the treatments at the Z2, Z3 and Z4 stage (Table 4). However, as the larvae developed to Z5 and megalopa stage, significant differences became evident. The highest survival was obtained when *Artemia* was introduced at Z3 (R6A and R6+A) or Z4 (R9A). When only rotifers were fed throughout the larval rearing period (R), zoeal development could be completed, however, survival and individual megalopa dry weight were significantly lower and duration of the zoeal stage was significantly longer ($P<0.05$). Early weaning onto *Artemia* at Z2 (R3A) or postponing it until Z5 (R12A) resulted in intermediate survival and individual megalopa dry weight. The latter treatment (R12A) moreover resulted in a significantly longer duration of the zoeal stage ($P<0.05$). Whilst no significant difference were observed for LSI among the treatments at DAH4 and DAH6 ($P>0.05$). At DAH10 LSI was significantly higher in treatments R3A, R6A and R6+A. At DAH15 and DAH19, LSI was higher in R3A, R6A, R9A and R6+A (Table 5).

Table 4. Survival percentage, individual megalopa dry weight and duration of the zoeal stage (LSI≥5.90) of *Eriocheir sinensis* subjected to different feeding schemes (Experiment I)

Treatment	Survival percentage (%)					Individual megalopa dry weight (μg)	Duration of zoeal stage (days)
	Z2	Z3	Z4	Z5	Megalopa		
R	90.7±5.7^{a}	73.6±2.4^{a}	52.1±7.9^{a}	13.3±3.9^{c}	6.2±1.8^{c}	425.0±5.0^{b}	22.2±1.2^{c}
R3A	92.7±2.8^{a}	73.2±1.9^{a}	68.9±4.9^{a}	28.6±6.3^{ab}	19.0±3.3^{ab}	465.0±33.8^{ab}	18.2±0.4^{a}
R6A	92.1±4.2^{a}	73.7±6.2^{a}	58.3±4.6^{a}	37.8±8.4^{a}	28.1±2.9^{a}	563.6±63.9^{a}	18.2±0.4^{a}
R9A	88.9±9.0^{a}	73.8±9.1^{a}	58.9±12.6^{a}	38.7±2.4^{a}	27.7±3.9^{a}	535.2±54.9^{a}	18.8±0.4^{a}
R12A	88.4±4.8^{a}	79.7±8.1^{a}	63.1±14.1^{a}	21.7±2.5^{bc}	16.7±3.4^{b}	491.4±38.6^{ab}	20.8±0.3^{b}
R6+A	89.1±4.2^{a}	70.7±7.7^{a}	64.2±7.1^{a}	37.6±7.7^{a}	28.0±5.5^{a}	548.9±48.4^{a}	18.2±0.4^{a}

Data are shown as mean ± standard deviation (n=6). Values in the same column showing the same superscript letter are not significantly different (P>0.05). Refer to Table 1 for treatment description

Table 5. Larval stage index (LSI) values of *Eriocheir sinensis* larvae subjected to different feeding schemes (Experiment I)

Treatment	Larval stage index (LSI)				
	DAH 4	DAH 6	DAH 10	DAH 15	DAH 19
R	2.00±0.01^{a}	2.99±0.01^{a}	3.71±0.22^{b}	4.62±0.21^{b}	5.21±0.19^{c}
R3A	2.00±0.01^{a}	2.98±0.01^{a}	3.94±0.03^{a}	5.00±0.03^{a}	6.00^{a}
R6A	1.99±0.01^{a}	2.96±0.02^{a}	3.90±0.03^{a}	4.99±0.01^{a}	6.00^{a}
R9A	2.00±0.01^{a}	2.99±0.01^{a}	3.61±0.07^{b}	4.96±0.03^{a}	5.94±0.03^{a}
R12A	1.99±0.01^{a}	2.98±0.02^{a}	3.65±0.09^{b}	4.74±0.09^{b}	5.62±0.18^{b}
R6+A	1.98±0.01^{a}	2.96±0.02^{a}	3.89±0.07^{a}	4.98±0.02^{a}	6.00^{a}

Data are shown as mean ± standard deviation (n=6). Values in the same column showing the same superscript letter are not significantly different (P>0.05). Refer to Table 1 for treatment description

3.2 Experiment II

Results of Experiment II are summarized in Table 6 and Table 7. Maximum survival at the Z2 and Z4 stage was recorded for TR5 (20/30/20 rotifers mL^{-1}). At the Z3, Z5 and megalopa stage, maximum survival was already attained in TR3 (10/15/10 rotifers mL^{-1}) (P<0.05). There were no obvious negative effects due to excess feeding levels (Table 6). The LSI increased significantly to a plateau in TR5 at DAH5 and DAH12, TR4 at DAH8 and DAH16, and TR3 at DAH19, with no further improvement beyond these levels (Table 7) (P<0.05). The equal amounts of *Artemia* fed in the later larval stages, seemed to some extent to have compensated for the low initial rotifer feeding rations (e.g. in TR3), therefore made result towards the end of the experiment less pronounced. However, extremely low rations as in TR1 (1/5/1 mL^{-1}) and TR2 (5/10/5 mL^{-1}) still resulted in a significant delay in larval development (in terms of LSI and duration of

zoeal stage), impaired survival and lower individual megalopa weight (the latter only in case of TR1) (P<0.05).

Table 6. Survival percentage, individual megalopa dry weight and duration of the zoeal stage (LSI≥5.90) of *Eriocheir sinensis* fed different densities of the rotifers from Z1 to Z3 stage, followed by the same density of *Artemia* feeding from Z3 to megalopa stage (Experiment II)

Treatment	Survival percentage (%)					Individual megalopa dry weight (μg)	Duration of zoeal stage (days)
	Z2	Z3	Z4	Z5	Megalopa		
TR1	38.3±5.7^{d}	27.6±4.7^{c}	20.0±3.9^{d}	18.3±4.2^{c}	15.2±3.1^{c}	548.3±36.0^{b}	20.0±0.6^{c}
TR2	55.0±6.0^{c}	40.1±5.4^{b}	34.6±4.6^{c}	32.3±3.8^{b}	24.3±3.4^{b}	593.3±30.8^{ab}	19.2±0.4^{b}
TR3	67.1±7.8^{b}	58.5±8.9^{a}	51.3±7.3^{b}	46.6±6.8^{a}	38.5±6.0^{a}	588.3±41.7^{ab}	18.2±0.4^{a}
TR4	74.0±6.1^{ab}	64.2±4.8^{a}	57.7±3.8^{ab}	49.2±4.0^{a}	43.0±3.5^{a}	619.6± 0.3^{a}	18.0±0.6^{a}
TR5	76.5±6.6^{a}	66.1±8.4^{a}	60.5±8.2^{a}	54.1±10.2^{a}	44.8±6.8^{a}	574.3±46.7^{ab}	18.2±0.4^{a}
TR6	69.4±5.9^{ab}	60.9±5.3^{a}	52.6±3.7^{ab}	46.6±5.3^{a}	38.9±4.4^{a}	601.7±11.7^{ab}	18.2±0.4^{a}
TR7	72.2±3.1^{ab}	60.1±4.6^{a}	53.3±8.1^{ab}	49.5±8.3^{a}	41.7±6.7^{a}	616.7±30.8^{a}	18.2±0.4^{a}

Data are shown as mean ± standard deviation (n=6). Values in the same column showing the same superscript letter are not significantly different (P>0.05). Refer to Table 2 for treatment description

Table 7. Larval stage index (LSI) values of *Eriocheir sinensis* larvae fed different densities of rotifers from Z1 to Z3 stage, followed by the same density of *Artemia* from Z3 to megalopa stage (Experiment II)

Treatment	Larval stage index (LSI)				
	DAH 5	DAH 8	DAH 12	DAH 16	DAH 19
TR1	1.48±0.05^{d}	2.46±0.05^{c}	3.84±0.03^{d}	4.97±0.05^{c}	5.86±0.05^{c}
TR2	1.64±0.07^{c}	2.74±0.06^{b}	3.93±0.09^{d}	5.02±0.04^{c}	5.90±0.02^{b}
TR3	1.79±0.09^{b}	2.81±0.08^{ab}	4.16±0.11^{c}	5.24±0.09^{b}	5.95±0.04^{a}
TR4	1.85±0.07^{ab}	2.86±0.08^{a}	4.36±0.10^{ab}	5.41±0.07^{a}	5.96±0.03^{a}
TR5	1.91±0.03^{a}	2.83±0.13^{a}	4.37±0.08^{a}	5.39±0.09^{a}	5.97±0.03^{a}
TR6	1.89±0.01^{a}	2.85±0.03^{a}	4.27±0.11^{abc}	5.33±0.09^{ab}	5.98±0.02^{a}
TR7	1.87±0.05^{a}	2.85±0.05^{a}	4.25±0.07^{bc}	5.32±0.07^{ab}	5.98±0.02^{a}

Data are shown as mean ± standard deviation (n=6). Values in the same column showing the same superscript letter are not significantly different (P>0.05). Refer to Table 2 for treatment description

3.3 Experiment III

Results for experiment III are presented in Table 8 and Table 9. A significantly lower survival in TA1 (0.5/0.7/1 mL^{-1}) and TA2 (1.5/2.5/4 mL^{-1}) appeared at the megalopa stage (P<0.05) (Table 8). TA1, TA2 and TA7 (20/25/30 mL^{-1}) moreover resulted in significantly longer durations of the zoeal stage (P<0.05). The highest individual megalopa weight was obtained in TA4 (6/10/15 mL^{-1}) and TA5 (10/15/20 mL^{-1})

(P<0.05). With increasing *Artemia* density, LSI increased significantly to a maximum in TA4 at DAH10, DAH12 and DAH16, in TA2 at DAH14 and TA3 (3/5/8 mL^{-1}) at DAH18, with no further improvements beyond these levels (P<0.05) (Table 9). Moreover, significantly lower LSI were observed in TA7 at DAH10, DAH14 and DAH16, and in TA5, TA6 and TA7 at DAH12 (P<0.05).

Table 8. Survival percentage, individual megalopa dry weight and duration of the zoeal stage (LSI≥5.90) of *Eriocheir sinensis* larvae fed different densities of *Artemia* from Z3 to megalopa stage (Experiment III)

Treatment	Survival percentage (%)			Individual megalopa dry weight (μg)	Duration of zoeal stage (days)
	Z4	Z5	Megalopa		
TA1	97.9±1.0^{a}	89.6±4.3^{a}	71.1±7.6^{b}	420.8±24.6^{d}	15.2±0.4^{c}
TA2	96.9±1.4^{a}	88.4±4.1^{a}	76.9±3.0^{b}	552.5±34.0^{c}	10.0±0.63^{b}
TA3	96.2±2.8^{a}	90.2±3.4^{a}	83.6±4.8^{a}	591.7±22.3^{bc}	9.7±0.5^{ab}
TA4	96.4±2.4^{a}	89.3±6.0^{a}	86.7±6.9^{a}	650.0±45.5^{a}	9.2±0.4^{a}
TA5	96.7±2.2^{a}	90.6±2.3^{a}	88.7±0.8^{a}	617.7±13.7^{ab}	9.2±0.4^{a}
TA6	93.8±4.1^{a}	89.9±2.1^{a}	86.9±1.4^{a}	586.7±30.8^{bc}	9.5±0.6^{a}
TA7	94.8±3.4^{a}	90.6±3.8^{a}	86.7±1.7^{a}	580.0±25.3^{bc}	10.0^{b}

Data are shown as mean ± standard deviation (n=6). Values in the same column showing the same superscript letter are not significantly different (P>0.05). Refer to Table 3 for treatment description

Table 9. Larval stage index (LSI) values of *Eriocheir sinensis* larvae fed different densities of *Artemia* from Z3 to megalopa stage (Experiment III)

Treatment	Larval stage index (LSI)				
	DAH 10	DAH 12	DAH 14	DAH 16	DAH 18
TA1	3.51±0.04 ^{c}	3.95±0.04 ^{d}	4.54±0.05 ^{b}	4.82±0.06 ^{d}	5.26±0.05 ^{c}
TA2	3.71±0.05 ^{ab}	4.20±0.07 ^{c}	4.95±0.08 ^{a}	5.32±0.09 ^{c}	5.93±0.07 ^{b}
TA3	3.72±0.05 ^{ab}	4.31±0.06 ^{bc}	4.99±0.02 ^{a}	5.47±0.05 ^{ab}	5.98±0.01 ^{a}
TA4	3.77±0.02 ^{a}	4.43±0.10 ^{a}	4.99±0.01 ^{a}	5.55±0.09 ^{a}	5.98±0.02 ^{a}
TA5	3.74±0.05 ^{a}	4.41±0.06 ^{ab}	4.99±0.01 ^{a}	5.53±0.10 ^{a}	5.98±0.03 ^{a}
TA6	3.74±0.04 ^{a}	4.28±0.09 ^{bc}	4.98±0.02 ^{a}	5.49±0.08 ^{a}	5.98±0.03 ^{a}
TA7	3.67±0.04 ^{b}	4.25±0.05 ^{c}	4.98±0.01 ^{b}	5.37±0.09 ^{bc}	5.99±0.01 ^{a}

Data are shown as mean ± standard deviation (n=6). Values in the same column showing the same superscript letter are not significantly different (P>0.05). Refer to Table 3 for treatment description

4. Discussion

Suitable prey for larvae should meet three general criteria: 1) appropriate size to be easily captured, digested and assimilated by the cultured species; 2) adequate

concentration to supply enough food, and on the other hand, to avoid detrimental effects of excess feeding 3) sufficient dietary nutrients (Sulkin, 1975; Ruscoe et al., 2004). Rotifers are considered as a good diet for early brachyuran larvae. Firstly, the size of rotifers appears to be ideal as food for the early zoeal stage larvae of many species, whose mouth parts are still too small to accept *Artemia* nauplii (Chang and Wu, 1985). Secondly, rotifers are also more digestible than *Artemia* (Watanabe et al., 1978; Dhert, 1996) as early stage brachyuran zoea larvae generally exhibit relatively low levels of enzyme activity and digestive capacity (Le Vay et al., 2001). Thirdly, early zoea larvae, particularly Z1, are passive feeders that do not actively pursue their prey (Heasman and Fielder, 1983), so the capture of highly mobile *Artemia* nauplii is more difficult than a slower swimming rotifer. On the other hand, *Artemi*a are more suitable to sustain development of later larval stages. Besides their bigger size, the lipid content of *Artemia* nauplii is 2-3 times as much as rotifers, which is particularly required by later zoeal development for metabolic activity and the production of hormones for the metamorphosis of brachyurans (Sulkin, 1975). Larger prey also results in a better feeding efficiency and can also help to avoid cannibalism.

Besides the suitable prey size, the strength of aeration is also a crucial factor in mitten crab larval rearing systems. In the early larval stages, particularly Z1 stage, a gentle aeration should be provided to avoid dorsal spine breaking. The larvae with broken spines are usually vulnerable to infestation by bacteria, viruses, fungi and ciliates, etc. On the other hand, at later larval stages, especially during the metamorphosis from Z5 to megalopa, a relatively strong aeration is required. It homogenizes the food in the water column, as well as disperses the larvae and therefore minimizes the interaction and physical contact between the animals, thus reducing cannibalism.

4.1 Optimum weaning time

Timing of live prey provision is considered as one of the most important feeding strategy aspects for brachyuran larvae, which hatch with little or no yolk reserve (Zeng and Li, 1992; Suprayudi et al., 2002). *Eriocheir sinensis* Z1 larvae can take in exogenous feed immediately after hatch, thus in the hatchery feed is generally provided

in advance in the rearing tanks.

The results from Experiment I demonstrate that, although *Eriocheir sinensis* zoea larvae could metamorphose to megalopa by only feeding on rotifers, significantly lower survival and individual megalopa dry weight, a low LSI and a long zoeal stage duration were obtained in this treatment. This is similar to what was observed for other brachyuran larvae, e.g. the blue crab *Callinectes sapidus* (Sulkin, 1975), the fiddler crab *Uca Pugilator* (Christiansen and Yang, 1976) and the mud crab *Scylla serrata* (Baylon and Failaman, 1997; Davis et al., 2005a). In the current study, though better than only rotifer feeding, delaying weaning onto *Artemia* up to the Z5 stage also led to lower megalopa survival, LSI and a longer duration of the zoeal stage. The inferior performance of *Eriocheir sinensis* larvae when fed only rotifers or when delaying weaning onto *Artemia* could be due to nutritional and energetic deficiencies when ingesting rotifers at later larval stages.

On the other hand, early weaning onto *Artemia* at the Z2 stage also resulted in inferior performance, which is probably due to the limited ability to capture *Artemia* and the low enzyme activity of early zoea larvae of *Eriocheir sinensis*. Z1 and Z2 larvae are not able to ingest *Artemia* in one piece because the prey is too large and possibly too difficult to break apart in smaller pieces. Furthermore a well developed hepatopancreas only appears from the Z3 stage onwards (Du et al., 1992), which is associated with a remarkable enhancement of enzyme activity of both pepsin and trypsin at this stage (Pan and Wang, 1997).

The results from Experiment I indicate that the optimal time to wean the larvae to *Artemia* was at the Z3 or Z4 stage, and that co-feeding with rotifers at Z3-Z4 stages seemed not really necessary since the larvae could adapt to the new food source quite easily. This is in agreement with studies on other brachyuran larvae, e.g. the mud crab *Scylla serrata* (Suprayudi et al., 2002), the swimming crab *Portunus trituberculatus* (Tekeuchi et al., 1999) and the mitten crab *Eriocheir sinensis* (Jiang et al., 2000), which stated that the diet should be supplemented with *Artemia* at the Z3 stage. Thus the

Z3-Z4 stage can be identified as a critical stage in the development of *Eriocheir sinensis,* both from a nutritional and prey size point of view as the larvae become more efficient and selective predators and show a strong preference for *Artemia* over rotifers (Davis et al., 2005b); beyond which the lack of suitable food organisms compromise the larval survival and development.

4.2 Optimum rotifer and Artemia feeding density

Prey density greatly affects larval survival and development in many brachyuran crustaceans. Experiment II and III showed that low feeding rations (both rotifers and *Artemia*) results in low survival and LSI associated with a prolonged duration of the zoeal stage and inferior individual megalopa dry weight; whilst higher prey densities produce high survival with a shorter inter-molt period and accelerated metamorphosis. In this respect, our results are in line with the observations on other brachyuran larvae (Heasman and Fielder, 1983; Zeng and Li, 1992; Minagawa and Murano, 1993a; 1993b; Suprayudi et al., 2002).

In the current study, larval performance reached a plateau at a certain specific live food density. We considered this density to be the optimal live food density. It should be noted that our experimental design was based on the principle of providing increasing prey density with larval development, with the actual prey density levels derived from studies on other brachyuran larvae (Minagawa and Murano, 1993a; 1993b; Baylon et al., 2004). However, it can also be argued that as larvae grow, their foraging ability increases substantially, and so the increased catching capability may render prey density increase unnecessary. This is particularly true with high feeding rations, for which prey seem always available in the rearing medium. Therefore it can be assumed that the optimal *Artemia* levels obtained in this study could in practice be lowered somewhat. This is also import in view of the observation that excess rotifer feeding levels in the early larval stages generally did not result in adverse effects, but excess *Artemia* feeding at later larval stages appeared to exert negative effects on larval performance. The reason could be that the bigger sized *Artemia* in the culture medium compete for oxygen and space with the larvae, release more metabolites and cause severe fouling, which

could have a detrimental impact on water quality and bacterial load, and hence influence the larval performance (Gopalakrishnan, 1976).

Also practical and economical considerations might be important to determine optimal feeding schedules and feeding rations. Rotifer production is often seen as a major expense in marine hatchery management due to its requirement for space and intensive labor, and unpredictable crashes (Suprayudi et al., 2002). On the other hand, *Artemia* is probably the most universal prey organism used in aquaculture because of its high nutritional value, size and convenience for use (Sorgeloos, 1980). However the fluctuating and usually high market price of *Artemia* cysts often limits their use in hatcheries. Therefore it should be mentioned that, in practice, optimal feeding regimes should, apart from growth and survival of the larvae, also consider the ease of use and the cost of the feed. In this respect, we advise a rotifer feeding density for Z1 and Z2 of 15 and 20 mL^{-1}, respectively; and an *Artemia* feeding density for Z3, Z4 and Z5 of 3, 5 and 8 mL^{-1}, respectively.

It is concluded that rotifers are the preferred live feed for mitten crab larvae at early stages (Z1-Z2) and *Artemia* appear to be better than rotifers for later stages. Rotifers should ideally be replaced with *Artemia* at the Z3-Z4 stage. The optimal rotifer feeding density for Z1 and Z2 would be 15 and 20 mL^{-1}, respectively; while a proper *Artemia* feeding density for Z3, Z4 and Z5 would be 3, 5 and 8 mL^{-1}, respectively. Though overall larval rearing techniques and larval rearing density are similar to what is used in commercial operations, it is worthy to mention that this study was performed at an experimental scale and further verification at production scale is recommended.

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Chapter V Section III

The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae

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Abstract

The effect of varying levels of dietary n-3 highly unsaturated fatty acid (HUFA) and docosahexaenoic acid/eicosapentaenoic acid (DHA/EPA) ratios on growth, survival and osmotic stress tolerance of *E. sinensis* zoea larvae was studied in two separate experiments. In experiment I, larvae were fed rotifers and *Artemia* enriched with ICES emulsions containing 0, 30 and 50 % total n-3 HUFA levels but with the same DHA/EPA ratio of 0.6. In experiment II, larvae were fed different combinations of enriched rotifers and *Artemia*, in which, rotifers were enriched with emulsions containing 30% total n-3 HUFA, but with different DHA/EPA ratio of 0.6, 2 and 4; while *Artemia* were enriched with the same emulsions, but DHA/EPA ratio of 0.6 and 4. In both experiments, un-enriched rotifers cultured on baker's yeast and newly-hatched *Artemia* nauplii were used as control diets. Larvae were fed rotifers at zoea 1 and zoea 2 stages; upon reaching zoea 3 stage, *Artemia* was introduced.

Experiment I revealed no significant effect of prey enrichment on the survival of megalopa among treatments, but higher total n-3 HUFA levels significantly enhanced larval development (larval stage index, LSI) and resulted in higher individual dry body weight of megalopa. Furthermore higher dietary n-3 HUFA levels also resulted in better tolerance to salinity stress. Experiment II indicated that at the same total n-3 HUFA level, larvae continuously receiving a low dietary DHA/EPA ratio had significantly lower survival at the megalopa stage and inferior individual body weight at the megalopa stage, but no negative effect was observed on larval development (LSI). The ability to endure salinity stress of zoea 3, zoea 5 and megalopa fed diets with higher DHA/EPA ratio was also improved.

Keywords *Eriocheir sinensis* larvae; n-3 HUFA; DHA/EPA ratio; osmotic stress tolerance

1. Introduction

China is the only country in the world where Chinese mitten crab *Eriocheir sinensis* larval rearing and farming are practiced. In 2004, annual aquaculture production reached about 500,000 metric ton, valued at 4 billion US$ (Yang and Zhang, 2005). A breakthrough in mass propagation in hatcheries of *E. sinensis* larvae was achieved in the early 1980's (Zhao, 1980). During the last two decades, substantial improvements of hatchery techniques have resulted in a rapid increase of larval availability. However, poor and variable survival of the larvae, particularly at molting of zoea 1 (Z1) to Z2 and metamorphosis of Z5 to megalopa stage, are still a major problem in *E. sinensis* larvae production (Zhou et al., 2000). Apart from ontogenetic and zootechnical factors, nutritional deficiencies or unbalances may contribute to the generally low larval survival.

As a catadromous species, *E. sinensis* spends most of its life time in freshwater and only migrates to the sea to reproduce. Before the benthic juveniles move upstream to freshwater, the pelagic larvae pass five zoea stages in seawater. In hatcheries, *E. sinensis* larvae are mostly cultured on a combination of microalgae, rotifers and *Artemia*. Rotifers and *Artemia* contain high levels of short chain saturated and mono-unsaturated fatty acids, but often lack n-3 highly unsaturated fatty acids (n-3 HUFA), such as eicosapentaenoic acid (EPA, 20:5n-3) (in rotifers) and docosahexaenoic (DHA, 22:6n-3) (in rotifers and *Artemia*) (Lavens and Sorgeloos, 1996). It is well known that marine crustaceans have a limited ability to elongate C18 n-3 poly unsaturated fatty acids (n-3 PUFA) to n-3 HUFA (Kanazawa and Teshima, 1977; Kanazawa et al., 1979; Suprayudi et al., 2004a), which therefore must be obtained from the feed, for example, through rotifer and *Artemia* enrichment. In contrast to marine species, freshwater species have a lower requirement for n-3 HUFA and a higher capacity to elongate and desaturate these from shorter chain fatty acids (Sargent et al., 1999). Because of the catadromous nature of *E. sinensis*, it is not clear whether it should be regarded as a freshwater or with marine species in aspects of lipid nutrition.

Dietary n-3 HUFA supplementation, mainly EPA and DHA have been reported to improve survival and/or growth of shrimp larvae and postlarvae, such as *Fenneropenaeus chinensis* (Xu et al., 1993), *Penaeus monodon* (Millamena et al., 1988; Rees et al., 1994), *Penaeus japonicus* (Kontara et al., 1997) and *Litopenaeus vannamei* (Léger and Sorgeloos, 1992; Lim et al., 1997; Wouters et al., 1997); as well as crab larvae, such as *Eurypanopeus depressus* (Levine and Sulkin, 1984), *Portunus trituberculatus* (Takeuchi et al., 1999) and *Scylla serrata* (Davis et al., 2003; Suprayudi et al., 2004b). Moreover, quantitative requirements for dietary n-3 HUFA have been extensively investigated. Kanazawa et al. (1979) suggested that a dietary provision of 1% (10 mg g^{-1} dw) n-3 HUFA could be considered as a minimal value for postlarval penaeids. Rees et al. (1994) reported that 12.55 mg g^{-1} dw n-3 HUFA in the enriched *Artemia* enhanced survival of *Penaeus monodon* postlarvae, while supplying very high n-3 HUFA levels (31.2 mg g^{-1} dw) to the postlarvae had no effect on the survival. Furthermore, dietary n-3 HUFA was also reported to have a positive effect on the ability of penaeid shrimp to resist stress conditions, such as osmotic shock (Tackaert et al., 1992; Rees et al., 1994; Palácios et al., 2004), temperature fluctuation (Chim et al., 2001) and ammonia (Martins et al., 2006). Although similar effects of dietary HUFA on the survival, growth and salinity stress tolerance of *E. sinensis* zoea larvae and juveniles were observed by Cheng et al. (1998c), Xin et al. (1999) and Chen et al. (2000), no studies to date have investigated the quantitative requirements of mitten crab larvae for n-3 HUFA and more specifically the DHA/EPA ratio.

The aim of this study was to determine whether boosting rotifers and *Artemia* with different levels of n-3 HUFA and DHA/EPA ratios would affect growth, survival, metamorphosis and osmotic stress tolerance of *E. sinensis* zoea larvae.

2. Materials and Methods

2.1 Broodstock management and egg incubation

The study was carried out at the Tianjin Modern Fishery Technology and Engineering Center, Tianjin, China. The breeders were selected from crab farms in Chongming,

Shanghai and Yangcheng Lake, Jiangsu, and were transported to the center in February 2004 and 2006, for experiment I and II, respectively. After acclimation to the indoor captive conditions for a week, the female and male crabs (in a ratio of 3:1) were then placed into mating tanks containing 20 g L^{-1} diluted seawater at 15° C. After spawning, 10 berried females were selected and transferred into 1×1×1 m fiberglass incubation tanks equipped with shelters. Crabs were fed fresh clam *Ruditapes variegata* once a day at 10% of the total body weight. Different incubation temperatures were used in both experiments. This was done for practical reasons in order to be able to spread the hatching of the larvae in time. In experiment I, low temperature incubation was applied through the use of an aquarium cooling machine. Water temperature was decreased gradually from 15° C to 8 ºC at a rate of 1 ºC per day. After 60 days incubation at 8° C, water temperature was increased gradually by 0.5° C per day until 21° C. In experiment II, high temperature incubation was conducted by increasing water temperature immediately after spawning, from 15° C to 21° C with a daily increase of 0.5° C. Once the incubation temperature reached 21° C, embryogenesis was observed daily under a microscope. Water was renewed every two days during this process. One day before the eggs were expected to hatch, the crabs were disinfected in a 0.2 g L^{-1} formalin bath for 30 min and transferred individually into 160 L plastic hatching tanks containing 20 g L^{-1} diluted seawater at 22 ºC. After the larvae were released, the spent crab was immediately removed from the hatching tank and the larvae were fed un-enriched rotifers *ad libitum*. One day after hatching (DAH1), positively photo-tactic Z1 larvae from a single breeder were selected and distributed randomly into the experimental larval rearing set-up.

2.2 Larval rearing

From DAH2 onwards, larvae were fed the different experimental diets twice a day (at 10 hrs and 22 hrs). The prey density was kept as following: Z1: 10 rotifers mL^{-1}; Z2: 15 rotifers mL^{-1}; Z3: 10 rotifers + 0.5 *Artemia* mL^{-1}; Z4: 1 *Artemia* mL^{-1}; Z5: 1.5 *Artemia* mL^{-1}.

Each treatment consisted of six replicate beakers containing 1.5 L diluted seawater stocked with 200 Z1 for the purpose of survival and larval stage index (LSI) determination; and four rectangular PVC tanks containing 20 L diluted seawater (20 g L^{-1}) at an approximate stocking density of 130 Z1 L^{-1} for assessing body weight, osmotic stress tolerance and tissue HUFA composition.

During the larval rearing period, water temperature ranged between 22 °C and 23 °C. Temperature in both beakers and PVC tanks was maintained in a heated water bath. Salinity was controlled at 20 g L^{-1}. pH ranged between 8.1 and 8.4. Aeration was gentle at Z1 stage and gradually increased at each stage to become relatively strong when Z5 molted into megalopa in order to prevent cannibalism. Photoperiod was maintained at 12L:12D with white fluorescent tubes.

From Z3 onwards, part of the water in the PVC tanks was renewed every morning to remove dead larvae, faeces, un-eaten rotifers and *Artemia*. The water renewal increased from 1/2 to 2/3 of the tank volume along with larval development. Each day the content of the plastic beakers was gently poured into a bowl and the surviving larvae counted while being pippetted into new beakers filled with new culture water. These new containers had been incubated in the water bath beforehand to adjust the water to the same temperature.

2.3 Rotifer and Artemia culture and enrichment

Rotifers (*Brachionus rotundiformis*) were cultured in a 100-L cylindrical PVC tank and fed baker's yeast. Rotifer enrichment was performed over a 3 h period at 33 °C, at a density of 2000 rotifers mL^{-1}, with a dose of 0.1 g emulsion L^{-1} according to the treatments outlined below. *Artemia* cysts (EG® type, INVE Aquaculture NV, Belgium) were hatched and harvested as described by Van Stappen (1996). The newly-hatched nauplii were enriched for 24 h at 28 ºC, at a density of 300 nauplii mL^{-1}, with doses of 0.3 g emulsion L^{-1} added at a 12 h interval. After enrichment, the animals were rinsed thoroughly with clean seawater to remove any remaining emulsion, and fed to the larvae. Rotifer enrichment was done twice a day (for each feeding). Both enriched metanauplii

and newly-hatched *Artemia* which could not immediately be fed to the larvae were kept at about 7 °C to minimize fatty acid catabolism and hence keep the nutritional value constant (Merchie, 1996).

The lipid emulsions used for live feed enrichment in this study were ICES Standard Reference Emulsions (ICES, 1997). Over the two experiments, five different live feed enrichment emulsions were used: ICES 0/-/c emulsion containing mainly saturated fatty acids; ICES 30/0.6/c and ICES 50/0.6/c emulsions containing 30% and 50% total n-3 HUFA, respectively, but with the same DHA/EPA ratio of 0.6; ICES 30/2/c and ICES 30/4/c both containing the same level of 30% total n-3 HUFA, but with DHA/EPA ratios of 2 and 4, respectively.

In experiment I, the rotifers and *Artemia* nauplii were enriched with emulsions containing 0, 30% and 50% of total n-3 HUFA levels, respectively:
Treatment 1 (T1): Rotifers + *Artemia* nauplii enriched with emulsion ICES 0/-/c
Treatment 2 (T2): Rotifers + *Artemia* nauplii enriched with emulsion ICES 30/0.6/c
Treatment 3 (T3): Rotifers + *Artemia* nauplii enriched with emulsion ICES 50/0.6/c
The control consisted of un-enriched rotifers and un-enriched newly-hatched *Artemia* nauplii.

In experiment II, the rotifers and *Artemia* nauplii were enriched with emulsions containing 30% of total n-3 HUFA, but varying DHA/EPA ratios: 0.6, 2 and 4, respectively:
Treatment 1 (T1): Rotifers enriched with 30/0.6/c + *Artemia* enriched with 30/0.6/c
Treatment 2 (T2): Rotifers enriched with 30/0.6/c + *Artemia* enriched with 30/4/c
Treatment 3 (T3): Rotifers enriched with 30/2/c + *Artemia* enriched with 30/0.6/c
Treatment 4 (T4): Rotifers enriched with 30/2/c + *Artemia* enriched with 30/4/c
Treatment 5 (T5): Rotifers enriched with 30/4/c + *Artemia* enriched with 30/0.6/c
Treatment 6 (T6): Rotifers enriched with 30/4/c + *Artemia* enriched with 30/4/c
The control consisted of un-enriched rotifers and un-enriched newly-hatched *Artemia* nauplii.

2.4 Biological parameters

2.4.1 Larval stage index and individual body weight

Larval development was monitored daily by identifying the stage of each larva in the beakers and assigning it a value: Z1=1, Z2=2, etc. to megalopa M=6. To compare larval development among treatments, an average larval stage index (LSI) in each treatment was then calculated as described by Millamena and Bangcaya (2001). At different larval stages, respectively 200 Z1, 100 Z3, 50 Z5 and 20 megalopa from each replicate PVC tank were sampled and rinsed thoroughly with distilled water, and the individual dry body weight was determined (60 °C, 24 h).

2.4.2 Survival

In both trials, the main experiment was stopped when all Z5 had metamorphosed to megalopa. Survival at that time was calculated on the initial number of Z1 (200 individuals). In experiment II, newly metamorphosed megalopa were however removed daily from the rearing containers to avoid cannibalism on the remaining Z5. These megalopa from each treatment were transferred to four plastic beakers containing 1500 mL diluted seawater (20 g L^{-1}) and fed the different enriched *Artemia* as before for a follow-up experiment. The survival of these megalopa after 3 days (DAH22) rearing was then determined.

2.4.3. Osmotic stress tolerance

In the different treatments, the osmotic stress tolerance of Z3, Z5 and megalopa was tested. Since resistance to osmotic stress is generally believed to increase with the age of crustacean larvae (Samocha et al., 1998), different salinities were used at Z3, Z5 and megalopa stage. The larvae were suddenly transferred from the normal rearing water (20 g L^{-1}) into water with a salinity of 45 g L^{-1}, 50 g L^{-1} and 60 g L^{-1}, respectively. A static system without aeration was applied. A group of 10 larvae from each replicate PVC tank was randomly sampled and transferred into plastic beakers containing 100 mL water. Four replicates were conducted for each treatment. Mortality was assessed at 10 min intervals. Larvae were considered dead when there was no movement of the appendages and when they did not respond to prodding with a glass pipette. Average

cumulative mortalities of the different treatments at different time intervals are reported. In addition a cumulative stress index (CSI) was calculated on the accumulative number of dead larvae at different time intervals as described by Dhert et al. (1992).

2.5 Chemical analysis

Rotifers and *Artemia* (before and after enrichment) were sampled on three separate occasions and pooled for lipid analysis. Z1, Z3, Z5 and megalopa from each treatment were also randomly sampled from each replicate tank and pooled together. After rinsing with distilled water, the samples were stored at -20 °C for later analysis. The total lipid contained in the live food and larvae were extracted according to Folch et al. (1957) using chloroform and methanol (2:1 v/v). The fatty acid composition was analyzed according to the modified procedure of Lepage and Roy (1984).

2.6 Statistical analysis

Data were presented as mean ± standard deviation and were subjected to statistical analysis using the software SPSS. Statistical significance of differences among treatments was determined using one-way ANOVA. Duncan multiple range test was applied to detect significant differences between means ($P<0.05$). Percentage data were arc-sin square root transformed prior to analysis.

3. Results

Table 1. Fatty acid composition of rotifers and *Artemia* nauplii enriched with emulsion containing 0, 30 and 50% n-3 HUFA with DHA/EPA ratio of 0.6 (Experiment I)

Fatty acids (mg g^{-1} dw)	Rotifers				*Artemia* nauplii			
	T1 0% n-3 HUFA	T2 30% n-3 HUFA	T3 50% n-3 HUFA	Control (un-enriched)	T1 0% n-3 HUFA	T2 30% n-3 HUFA	T3 50% n-3 HUFA	Control (un-enriched)
16:0	8.52	10.61	7.87	5.79	12.70	15.78	12.44	15.11
16:1 n-7	5.90	8.13	5.93	6.10	2.74	5.22	6.69	3.89
17:0	0.61	0.87	0.62	0.58	0.66	1.01	1.90	0.90
17:1 n-7	0.59	0.42	0.78	0.63	0.80	1.00	1.15	1.12
18:0	3.41	3.48	3.73	2.50	6.81	7.59	7.74	6.37
18:1n-9+ n-7	8.29	10.01	9.17	5.76	28.31	34.22	36.40	34.02
18:2 n-6	5.24	4.63	4.81	3.34	7.32	8.01	10.13	9.01
18:3 n-6	0.40	0.46	0.50	0.49	0.66	0.85	1.17	1.12
18:3 n-3	2.06	2.11	2.16	2.01	26.90	30.55	33.14	41.25
18:4 n-3	0.43	1.10	0.94	0.40	4.53	4.83	5.43	7.12
20:4 n-6	0.67	0.92	1.52	0.58	1.01	1.37	4.64	1.01
20:4 n-3	2.41	2.46	3.01	2.42	0.68	1.10	1.92	1.14
20:5 n-3	3.29	8.11	13.40	1.31	2.97	12.12	19.94	3.06
22:1 n-9+ n-7	0.94	1.22	2.07	0.92	0.10	0.11	1.09	0.10
21:5 n-3	0.10	0.26	0.63	0.10	-	0.29	0.29	-
22:5 n-3	0.82	1.51	1.58	0.68	-	0.84	0.69	-
22:6 n-3	-	4.73	9.02	-	-	3.87	7.15	-
Total n-3 HUFA (n≥20)	6.22	17.07	27.64	3.01	3.65	18.22	29.99	4.20
Total n-6 HUFA (n≥18)	6.31	6.01	6.83	4.41	8.99	10.23	15.94	11.14
DHA/EPA	-	0.58	0.67	-	-	0.32	0.36	-
Total lipid (% dw)	15.21	15.49	18.90	4.83	16.40	18.72	21.71	14.37

3.1 Experiment I

The enrichment notably affected the total n-3 HUFA content in the rotifers and *Artemia* (Table 1). The rotifers and *Artemia* enriched with ICES 0/-/c (T1) contained low total n-3 HUFA levels (6.22 mg g^{-1} dw in rotifers, 3.65 mg g^{-1} dw in *Artemia*), which was

similar to the un-enriched ones (3.01 mg g^{-1} dw in rotifers, 4.20 mg g^{-1} dw in *Artemia*). On the other hand, the rotifers and *Artemia* enriched with ICES 30/0.6/c (T2) and ICES 50/0.6/c (T3) contained much higher levels of total n-3 HUFA (17.07 and 27.64 mg g^{-1} dw in rotifers, 18.22 and 29.99 mg g^{-1} dw in *Artemia,* respectively). Generally, when using the same emulsion, the DHA/EPA ratio in the enriched rotifers was higher than that in the enriched *Artemia,* (0.58 and 0.67 in T2 and T3 rotifers, 0.32 and 0.36 in T2 and T3 *Artemia,* respectively). Total n-6 HUFA content in rotifers and *Artemia* of the different treatments remained relatively stable.

The fatty acid composition of Z1, Z3, Z5 and megalopa is shown in Table 2. The total n-3 HUFA levels and DHA/EPA ratios of Z3, Z5 and megalopa reflected to some extent the levels in the enriched rotifers and *Artemia* with increasing total n-3 HUFA, DHA and EPA levels from T1 to T3. Both Z5 and megalopa generally contained higher levels of total n-3 HUFA (ranging from 4.92 to 20.50 mg g^{-1}dw for Z5, from 4.91 to 20.61 mg g^{-1}dw for megalopa) than Z3 (ranging from 7.10 to 12.39 mg g^{-1}dw), which mostly came from higher EPA levels in the body tissue.

No statistically significant differences were observed in megalopa survival among any of the treatments (Table 3). However, the megalopa receiving higher dietary n-3 HUFA levels (T2 and T3) had a higher LSI and individual body weight than those in T1 and the control group (P<0.05). When being abruptly transferred from 20 g L^{-1} culture water to 60 g L^{-1} brine water, the trend of cumulative mortality of megalopa was influenced by the dietary n-3 HUFA level. The cumulative stress index (CSI) at the megalopa stage was significantly affected by the dietary n-3 HUFA level, with increasing stress sensitivity in the order T3, T2, T1 and the control group (P<0.05) (Fig.1 and Table 3).

Table 2. Fatty acid composition and total lipid content of *E. sinensis* Z3, Z5 and megalopa fed rotifers and *Artemia* containing different total n-3 HUFA levels (Experiment I)

Fatty acids (mg g^{-1}dw)	Z 1	Z3			Z5				Megalopa				
		T1 0% n-3 HUFA	T2 30% n-3 HUFA	T3 50% n-3 HUFA	Control (un-enriched)	T1 0% n-3 HUFA	T2 30% n-3 HUFA	T3 50% n-3 HUFA	Control (un-enriched)	T1 0% n-3 HUFA	T2 30% n-3 HUFA	T3 50% n-3 HUFA	Control (un-enriched)
16:0	4.42	4.85	5.19	5.07	3.41	5.81	7.20	7.32	5.10	7.39	8.37	6.80	4.64
16:1 n-7	1.93	1.40	1.58	1.57	0.89	2.87	5.35	3.88	2.91	4.37	6.00	3.83	2.21
17:0	-	-	-	-	-	-	-	-	-	-	-	-	-
17:1 n-7	-	-	-	-	-	-	-	-	-	-	-	-	-
18:0	2.08	3.69	3.62	3.69	2.92	3.38	4.64	4.72	3.58	4.07	4.25	4.22	-
18:1 n-9 + n-7	16.07	9.26	8.88	9.68	5.98	16.68	25.19	22.71	18.12	22.40	25.76	22.71	15.77
18:2 n-6	5.31	3.51	2.62	2.83	2.48	5.12	6.08	5.27	3.78	6.36	5.94	5.15	2.21
18:3 n-6	-	-	-	-	-	-	-	-	-	-	-	-	-
18:3 n-3	0.93	5.16	1.91	5.87	3.11	10.34	14.38	13.47	10.72	13.04	13.36	13.12	7.53
18:4 n-3	0.84	0.00	0.00	0.00	0.25	1.75	2.77	2.56	1.63	1.97	2.29	2.28	0.90
20:4 n-6	3.81	3.10	2.58	3.12	2.81	2.95	3.57	4.21	3.52	3.38	3.37	4.26	3.27
20:4 n-3	-	-	-	-	-	-	-	-	-	-	-	-	-
20:5 n-3	4.53	5.47	7.39	8.37	4.79	4.19	13.17	15.24	5.93	4.34	12.87	15.39	5.61
22:1 n-9 + n-7	-	0.95	0.65	0.84	0.91	0.51	0.65	0.75	0.49	0.56	0.76	0.86	0.22
21:5 n-3	-	-	-	-	-	-	-	-	-	-	-	-	-
22:5 n-3	-	-	-	-	-	-	-	-	-	-	-	-	-
22:6 n-3	3.26	1.63	3.43	4.01	1.50	0.74	4.73	5.26	1.00	0.57	4.09	5.22	0.71
Total n-3 HUFA (n≥20)	7.80	7.10	10.82	12.39	6.28	4.92	17.90	20.50	6.93	4.91	16.96	20.61	6.32
Total n-6 HUFA (n≥18)	9.12	6.61	5.20	5.96	5.29	8.06	9.65	9.48	7.30	9.74	9.31	9.42	5.48
DHA/EPA	0.72	0.30	0.46	0.48	0.31	0.18	0.36	0.35	0.17	0.13	0.32	0.34	0.13

Table 3. Average cumulative stress index (CSI), larval stage index (LSI), individual dry body weight, survival of *E. sinensis* megalopa at DAH 16 fed rotifers and *Artemia* nauplii containing different total n-3 HUFA levels (Experiment I)

Treatments	Larval stage index (LSI)	Individual dry body weight (μg)	Survival (%)	Cumulative stress index (CSI)
T1 (0% n-3 HUFA)	5.83 ± 0.12^{bc}	957 ± 90^{b}	20.0 ± 6.1^{a}	69 ± 3^{b}
T2 (30%n-3 HUFA)	5.92 ± 0.11^{ab}	1203 ± 71^{a}	22.0 ± 6.6^{a}	62 ± 6^{ab}
T3 (50%n-3 HUFA)	5.99 ± 0.02^{a}	1203 ± 90^{a}	18.2 ± 5.6^{a}	57 ± 5^{a}
Control	5.74 ± 0.09^{c}	973 ± 25^{b}	24.7 ± 7.9^{a}	85 ± 7^{c}

Values are means ± S.D. Values in the same column showing the same superscript are not significantly different (P>0.05; n=6)

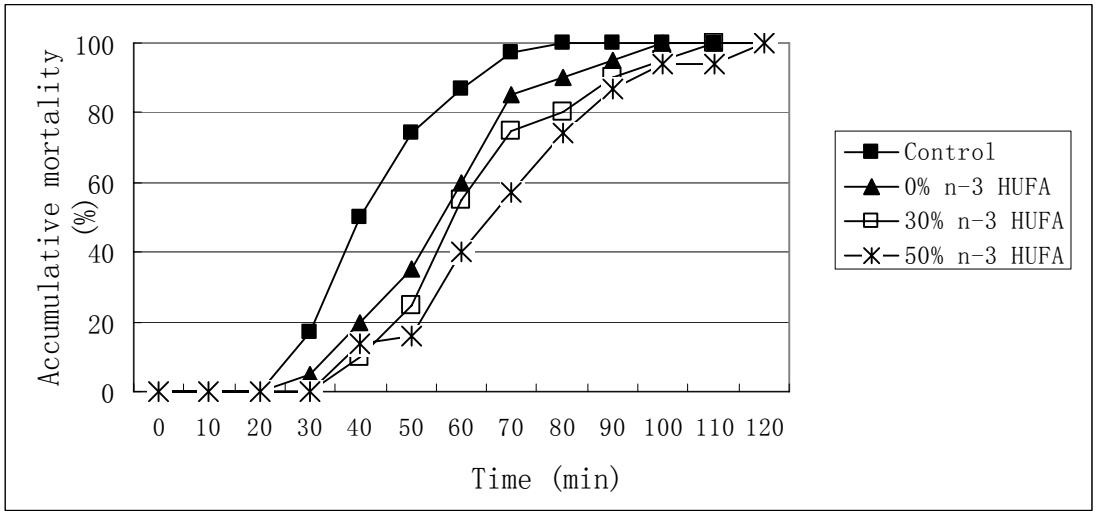

Fig. 1. Time-course of cumulative mortalities recorded when *E. sinensis* megalopa fed rotifers and *Artemia* nauplii containing different total n-3 HUFA levels were transferred from seawater with a salinity of 20 g L^{-1} to 60 g L^{-1} (n=4) (Experiment I)

3.2 Experiment II

The fatty acid composition of the un-enriched and enriched rotifers and *Artemia* is shown in Table 4. The total n-3 HUFA level in the enriched rotifers and *Artemia* (approximate 20 mg g^{-1} dw) were much higher than those of the un-enriched ones (less than 5 mg g^{-1} dw). The DHA/EPA ratios in the enriched rotifers and *Artemia* reflected those of the emulsions, ranging from 0.68 to 2.85 in rotifers and 0.28 to 0.96 in *Artemia*, respectively, and were much higher than in the un-enriched rotifers and *Artemia* (0.02 and 0.06, respectively). Similarly to the results of experiment I, the DHA/EPA ratio in rotifers was higher than in *Artemia* when enriched with the same emulsion.

The fatty acid composition of Z1, Z3, Z5 and megalopa is shown in Table 5. The total n-3 HUFA levels and DHA/EPA ratios of Z3 reflected the values of the enriched rotifers; while those of Z5 and megalopa reflected the values of the enriched *Artemia*, regardless of the type of rotifers they were fed on previously. Z5 fed with *Artemia* enriched with lower DHA/EPA ratio (0.6) emulsion, generally contained higher levels of total n-3 HUFA (ranging from 26.97 to 28.77 mg g^{-1}dw) than those receiving *Artemia* enriched with higher DHA/EPA ratio (4) emulsion (ranging from 18.48 to 22.47 mg g^{-1}dw), which were mostly contributed by higher EPA levels in the former treatments. However, similar n-3 HUFA levels (ranging from 21.06 to 22.81 mg g^{-1}dw) were observed at the megalopa stage. Metamorphosis from Z5 to megalopa resulted in all treatments in a slight drop of the DHA/EPA ratios and total n-3 HUFA levels. In the control group (un-enriched rotifers and newly-hatched *Artemia* nauplii), the total n-3 HUFA content of the larvae dropped substantially from 10.01 mg g^{-1}dw in Z1 to 5.21 mg g^{-1}dw in Z3, but tended to stabilize in Z5 and megalopa (5.78 and 6.10 mg g^{-1}dw, respectively).

Table 4. Fatty acid composition of rotifers and *Artemia* nauplii enriched with emulsions containing 30% total n-3 HUFA and with DHA/EPA ratio of 0.6, 2 and 4 (Experiment II)

Fatty acids (mg g^{-1} dw)	Rotifers				*Artemia* nauplii			
	DHA/ EPA 0.6	DHA/ EPA 2	DHA/ EPA 4	Un-enriched	DHA/ EPA 0.6	DHA/ EPA 2	DHA/ EPA 4	Un-enriched
16:0	15.48	15.23	14.98	4.91	17.93	17.15	16.22	14.44
16:1 n-7	17.89	17.09	14.01	16.16	9.85	8.40	8.43	7.72
17:0	1.24	1.44	2.17	-	2.44	2.34	2.66	1.85
17:1 n-7	0.87	0.91	0.73	0.47	1.40	1.25	1.42	1.35
18:0	7.14	6.74	4.09	2.91	10.26	10.09	8.77	7.07
18:1 n-9 + n-7	31.23	30.95	29.61	22.10	46.91	43.50	41.67	35.53
18:2 n-6	8.54	8.21	8.69	3.81	12.10	11.47	10.67	10.35
18:3 n-6	-	-	-	0.78	0.82	0.88	0.95	1.19
18:3 n-3	1.05	1.00	1.05	0.45	36.76	34.09	32.91	40.41
18:4 n-3	1.98	2.10	3.18	2.01	5.29	5.34	5.65	7.45
20:4 n-6	1.23	1.42	2.01	0.78	3.06	4.56	5.33	2.99
20:4 n-3	1.32	1.32	1.10	1.20	0.88	1.10	1.37	1.17
20:5 n-3	9.02	6.98	4.05	0.20	12.81	11.09	8.93	2.14
22:1 n-9 + n-7	0.21	0.20	1.05	0.19	0.17	0.50	1.10	0.09
21:5 n-3	1.45	1.25	1.70	0.49	1.25	1.89	0.63	0.66
22:5 n-3	1.63	1.73	0.84	0.48	1.82	1.39	0.67	0.41
22:6 n-3	6.09	8.17	11.52	0.23	3.56	6.23	8.25	0.13
Total n-3 HUFA (n≥20)	19.51	19.45	19.21	2.37	20.32	21.70	19.85	4.51
Total n-6 HUFA (n≥18)	9.77	9.62	10.71	5.60	15.97	16.91	16.94	14.54
DHA/EPA	0.68	1.17	2.85	0.02	0.28	0.56	0.92	0.06
Total lipid (% dw)	15.58	15.32	15.97	4.21	18.62	19.37	20.44	14.37

Table 5. Fatty acid composition and total lipid content of *E. sinensis* Z3, Z5 and megalopa fed rotifers and *Artemia* nauplii containing different DHA/EPA ratios (Experiment II)

Fatty acids (mg. g^{-1}dw)	Z 1	Z 3				Z 5							Megalopa						
		T1/T2 DHA/EPA 0.6	T3/T4 DHA/EPA 2	T5/T6 DHA/EPA 4	Control	T1 DHA/EPA 0.6/0.6	T2 DHA/EPA 0.6/4	T3 DHA/EPA 2/0.6	T4 DHA/EPA 2/4	T5 DHA/EPA 4/0.6	T6 DHA/EPA 4/4	Control	T1 DHA/EPA 0.6/0.6	T2 DHA/EPA 0.6/4	T3 DHA/EPA 2/0.6	T4 DHA/EPA 2/4	T5 DHA/EPA 4/0.6	T6 DHA/EPA 4/4	Control
16:0	5.39	6.41	6.52	6.31	4.27	11.49	7.10	10.81	7.66	10.12	8.07	8.35	9.48	10.05	9.15	9.88	9.63	10.31	7.69
16:1 n-7	3.26	3.50	3.45	3.49	3.79	6.27	2.80	5.70	3.33	5.32	3.10	3.52	5.32	4.91	5.34	4.76	5.60	5.09	3.07
17:0	0.45	0.57	0.76	1.05	-	1.64	1.08	1.38	1.17	1.28	1.26	0.97	1.21	1.54	1.19	1.53	1.29	1.56	0.79
17:1 n-7	0.22	-	0.22	0.16	-	0.84	0.49	0.81	0.58	0.81	0.63	0.53	0.83	0.90	0.86	0.93	0.85	1.02	0.46
18:0	2.87	4.74	4.55	4.29	3.98	6.18	4.23	5.89	4.35	5.55	4.67	4.53	4.73	4.73	4.54	4.80	4.66	5.13	4.04
18:1 n-9 + n-7	9.75	11.67	12.09	11.70	13.13	31.45	17.40	28.37	20.02	27.07	20.08	22.60	27.87	29.14	27.22	29.36	28.73	30.82	20.31
18:2 n-6	3.28	3.68	3.60	3.61	3.35	7.73	3.99	6.99	4.43	6.59	4.47	5.62	6.13	6.34	6.30	6.38	6.77	6.53	4.94
18:3 n-6	-	-	-	-	0.16	-	-	-	-	-	-	0.33	0.09	-	-	-	-	-	0.08
18:3 n-3	0.61	0.89	0.53	0.59	0.65	17.51	9.25	15.38	9.46	14.58	9.25	19.64	15.86	15.84	15.18	15.77	16.59	16.60	17.67
18:4 n-3	0.60	1.95	1.11	1.28	1.15	3.26	1.38	2.95	0.94	2.90	1.04	2.60	2.58	2.38	2.60	2.30	3.10	2.03	2.33
20:4 n-6	2.35	2.30	2.39	2.28	1.83	4.64	3.71	4.27	3.92	4.02	4.52	3.70	3.61	4.53	3.63	5.64	3.59	4.74	3.95
20:4 n-3	0.02	0.85	0.40	0.27	-	1.15	0.50	1.07	0.49	1.01	0.69	0.93	0.95	0.63	0.97	0.79	0.91	1.26	1.07
20:5 n-3	5.54	8.96	7.73	6.95	3.37	18.00	8.80	17.19	9.07	16.18	9.79	3.83	13.55	10.32	13.48	10.49	14.16	11.16	3.95
22:1 n-9 + n-7	0.92	0.50	0.40	0.12	-	0.75	0.45	0.57	0.33	1.02	0.96	0.93	0.98	0.53	0.55	0.70	0.91	0.59	0.12
21:5 n-3	0.47	0.79	0.51	0.15	-	0.57	0.57	0.27	0.73	0.90	2.04	0.34	0.26	1.18	1.13	0.85	0.62	0.98	0.06
22:5 n-3	0.25	0.86	0.63	0.73	-	1.40	0.55	1.24	0.54	1.23	0.74	0.13	1.07	0.71	1.15	0.63	1.16	0.73	0.60
22:6 n-3	3.73	6.04	6.85	6.41	1.84	7.29	8.06	7.87	8.06	7.65	9.20	0.54	5.24	9.00	5.82	8.55	5.96	8.68	0.32
Total n-3 HUFA (n≥20)	9.99	17.50	16.13	14.50	5.21	28.77	18.48	27.65	18.88	26.97	22.47	5.78	21.06	21.84	22.54	21.30	22.81	22.80	6.10
Total n-6 HUFA (n≥18)	5.64	5.98	6.00	5.89	5.34	12.37	7.70	11.27	8.35	10.61	8.99	9.66	9.83	10.87	9.94	12.01	10.36	11.26	8.97
DHA/EPA	0.67	0.67	0.89	0.92	0.55	0.41	0.92	0.46	0.89	0.47	0.94	0.14	0.39	0.87	0.43	0.82	0.42	0.78	0.08
Total lipid (% dw)	5.93	4.49	4.25	4.19	3.17	15.27	11.75	14.15	11.33	15.23	13.59	10.03	16.68	17.57	16.71	17.43	15.38	17.64	11.28

A significantly lower LSI was observed for the control group (P<0.05), whilst the other treatments in which the larvae received enriched diets gave similar results (Table 6). On the other hand, megalopa in T6 had a significantly higher body weight than those in T1 and the control group (P<0.05). Larvae continuously fed prey enriched with a low dietary DHA/EPA ratio (T1) had significantly lower survival at the megalopa stage (P<0.05), similar to the control group. Ongrowing of the megalopa for another 3 days only showed a significant difference in survival between the enrichment groups and the control group (P<0.05). Survival in the latter was around 15% lower.

Table 6. Average cumulative stress index (LSI) at Z3, Z5 and megalopa stage, larval stage index (LSI), individual dry body weight and survival at DAH 19 and ongrown at DAH 22 of *E. sinensis* megalopa fed rotifers and *Artemia* nauplii containing different DHA/EPA ratios (Experiment II)

Treatments	Larval stage index (LSI)	Individual dry body weight (μg)	Survival (%) (DAH19)	Survival (%) (ongrown)	Cumulative stress index (CSI)		
					Z3	Z5	Megalopa
T1 DHA/EPA (0.6/0.6)	5.93±0.07^{a}	487 ± 40^{bc}	52.3±6.2^{b}	73.3 ± 4.0^{a}	41 ± 4^{a}	80 ± 6^{c}	31 ± 1^{b}
T2 DHA/EPA (0.6/4)	5.99±0.02^{a}	563 ± 12^{ab}	61.9±6.0^{a}	76.2 ±2.7^{a}		55 ± 3^{b}	26 ± 2^{a}
T3 DHA/EPA (2/0.6)	5.93±0.10^{a}	533 ± 42^{ab}	67.5±3.8^{a}	73.2± 4.0^{a}	38 ± 4^{a}	56 ± 7^{b}	27 ± 3^{ab}
T4 DHA/EPA (2/4)	5.94±0.06^{a}	553 ± 40^{ab}	65.0±6.8^{a}	72.3 ± 5.2^{a}		55 ± 7^{b}	26 ± 1^{a}
T5 DHA/EPA (4/0.6)	5.94±0.08^{a}	577 ± 64^{ab}	65.2±7.3^{a}	77.2 ± 2.6^{a}	35 ± 6^{a}	53 ± 9^{b}	31 ± 5^{b}
T6 DHA/EPA (4/4)	5.93±0.07^{a}	613 ± 78^{a}	66.5±4.9^{a}	75.4 ± 7.1^{a}		38 ± 7^{a}	25 ± 3^{a}
Control	5.80±0.14^{b}	423 ± 21^{c}	50.0±5.9^{b}	58.4 ± 4.2^{b}	41 ± 2^{a}	91 ± 6^{d}	50^{c}

Values are means ± S.D. Values in the same column showing the same superscript are not significantly different (P>0.05; n=6)

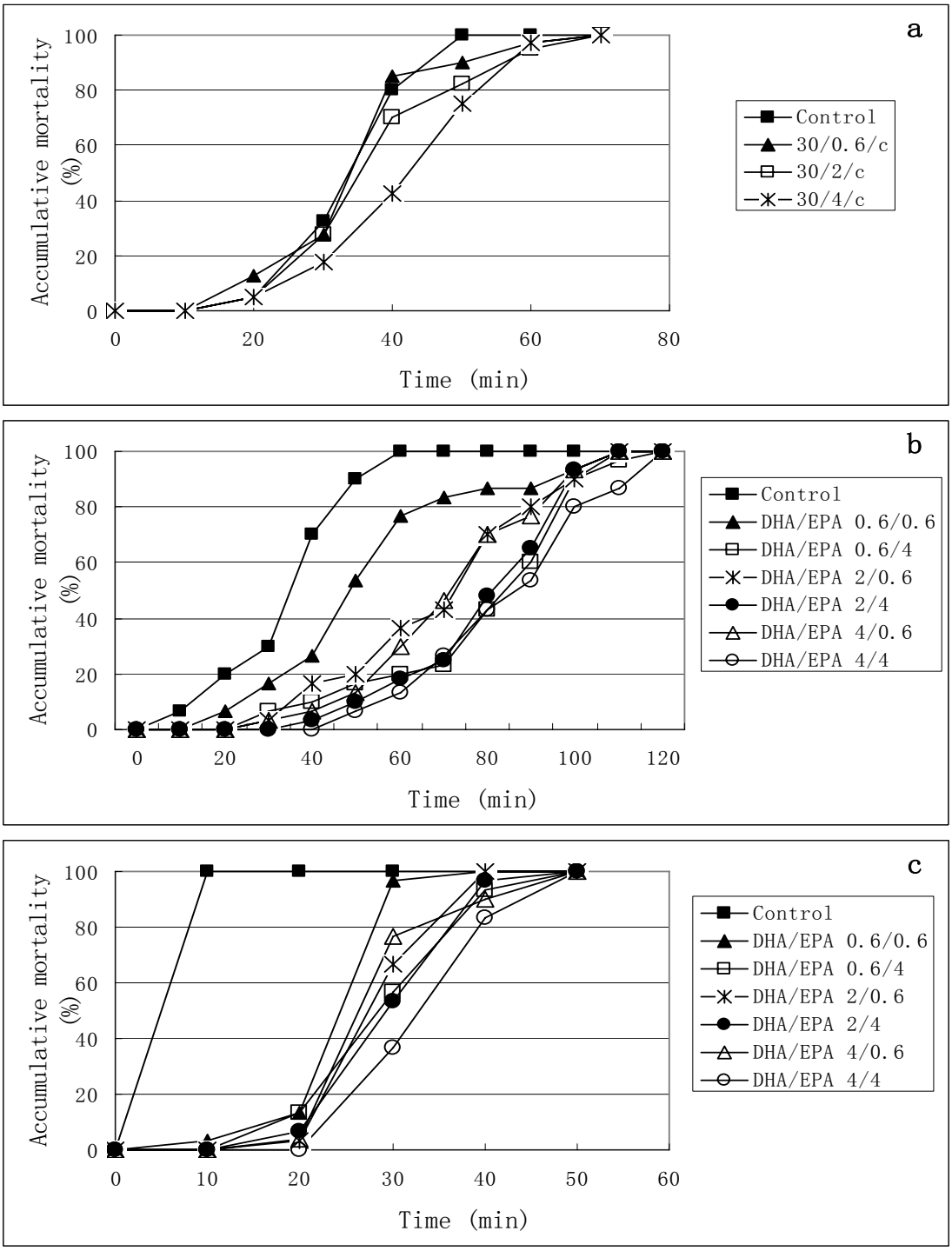

Fig. 2. Time-course of cumulative mortalities recorded when *E. sinensis* larvae fed rotifers and *Artemia* nauplii containing different DHA/EPA ratios were transferred from diluted seawater to different water medium (n=4). a) Z3: from 20 g L^{-1} to 45 g L^{-1}; b) Z5: from 20 g L^{-1} to 50 g L^{-1}; c) Megalopa: from 20 g L^{-1} to 60 g L^{-1} (Experiment II)

The osmotic stress tolerance of Z3, Z5 and megalopa is shown in Table 6 and Fig. 2a, 2b and 2c, respectively. When Z3 were abruptly transferred from 20 g L^{-1} culture water to 45 g L^{-1} brine water, there was a trend that a higher dietary DHA/EPA ratio resulted in better tolerance to salinity shock, especially for the DHA/EPA ratio of 4. The CSI was however not statistically different among the treatments. There was a clear tendency, however, that when Z5 were transferred directly from 20 g L^{-1} culture water to 50 g L^{-1} brine water, higher dietary DHA/EPA ratio resulted in markedly lower CSI (P<0.05). A similar trend was also observed when megalopa were transferred to 60 g L^{-1} brine water. Megalopa which received diets with higher DHA/EPA ratio in the *Artemia* had significantly lower CSI than those received diets with lower DHA/EPA ratio in the *Artemia*, despite of the DHA/EPA ratio in the rotifers that they received in early stages. This means that, even when larvae were fed rotifers enriched with a low DHA/EPA ratio in early stages, they could still recuperate when being fed with *Artemia* enriched with a high DHA/EPA ratio and giving improved results over the other treatments by the end of the culture period.

4. Discussion

As reported earlier by Dhert et al. (1993) and Sorgeloos et al. (2001), remarkable differences were observed in the possibility to boost the DHA/EPA ratio in rotifers and *Artemia*. Owing to the lower DHA and higher EPA retention in *Artemia*, lower DHA/EPA ratios were obtained after enrichment in *Artemia* compared with rotifers. Similar observations on DHA/EPA dynamics were noted by Wouters et al. (1997). This could be explained by the different life cycle of both species. Most of the rotifers are in adult stage where metabolic activities are not as pronounced as in the larval *Artemia* stage. As a consequence the fatty acid composition of the rotifers is more stable and does not change considerably during enrichment or starvation conditions. *Artemia* nauplii, on the other hand, do not synthesize DHA but metabolize HUFA very fast. Therefore the composition of *Artemia* nauplii is far more difficult to manipulate (Dhert et al., 1993). Nevertheless huge differences in the fatty acid composition were observed between the different enriched live preys and therefore the theoretical design of the

experiment was achieved.

When conducting larval nutrition studies, it is necessary to minimize the "masking effect" of other zootechnical factors, such as larval density, feeding strategy, water quality parameters, etc. This is of particular importance for carnivorous species, as this could significantly influence the survival. Metamorphosis from Z5 to megalopa is indeed a crucial stage for *E. sinensis* because of the cannibalistic behavior of megalopa (Yu Xuequan, personnel comm.). In Experiment I, cannibalism was evidently observed during metamorphosis from Z5 to megalopa, resulting in a drop in survival of about 20%. However, in experiment II, the influence of cannibalism on survival could be minimized by removing newly-metamorphosed megalopa from the rearing system. The survival obtained in Experiment II should therefore be a better reflection of the n-3 HUFA requirement of *E. sinensis* larvae. The inferior survival obtained with low dietary n-3 HUFA in control group in experiment II might indicate the importance of n-3 HUFA on the survival of *E. sinensis* larvae.

The results from Experiment I suggest that the low dietary n-3 HUFA level in T1 and the control group (6.22 and 3.01 mg g^{-1} dw in rotifers, 3.65 and 4.20 mg g^{-1} dw in *Artemia*), resulted in DHA depletion in the body tissues of the larvae, an inferior growth in terms of individual body weight and larval development, compared to T2 in which the live prey were boosted with n-3 HUFA (17.07 mg g^{-1} dw in rotifers and 18.22 mg g^{-1} dw in *Artemia*). A further increase in the dietary n-3 HUFA levels in T3 (27.64 mg g^{-1} dw in rotifers and 29.99 mg g^{-1} dw in *Artemia*) did not improve growth. Therefore we might suggest that 17 to 18 mg g^{-1} dw n-3 HUFA in the live food might be optimal for *E. sinensis* larval growth, and no deleterious effects of excess dietary n-3 HUFA were observed within the test range. These data are in accordance with a similar study in the swimming crab *Portunus trituberculatus,* for which the suitable range of n-3 HUFA in rotifers was between 9 to 17 mg g^{-1} dw (Takeuchi et al., 1999).

Apart from the quantitative requirements for dietary n-3 HUFA, the proportion of

specific fatty acids, e.g. DHA and EPA, is known to be a critical nutritional factor. It has been observed that developing eggs and larvae of marine fish preferentially consume DHA over EPA as a more efficient energy source or as precursors for prostaglandins (Watanabe, 1978), and DHA was superior to EPA in larval development of mud crab (*Scylla serrata*) (Suprayudi et al., 2004a). Although *E. sinensis* spend most of their life time in freshwater, larval development does take place in a marine/brackish environment; hence one could expect their requirements to be close to marine organisms in that they require a high DHA/EPA ratio in the diet. In Experiment II, a low DHA/EPA ratio in the T1 diet (0.68 in rotifers and 0.28 in *Artemia*, respectively) resulted in significantly inferior larval development (LSI), and lower megalopa survival than treatments with higher dietary DHA/EPA ratios (T2 to T6, ranging from 1.17 and 2.85 in rotifers, and 0.56 to 0.92 in *Artemia*). However, no significant differences were obtained in between the latter treatments. Thus we speculate that the DHA/EPA ratio of 1.17 in rotifers and 0.56 in *Artemia* is sufficient for optimal growth, survival and metamorphosis of *E. sinensis* larvae. This is somehow lower than the optimum ratio for marine fish species (in the range of 2:1) reported by Sargent et al. (1999). Meanwhile no negative effects of elevated dietary DHA/EPA ratios were observed within the range tested in this study.

The current study confirms earlier findings that crustacean larvae fed nutritionally balanced n-3 HUFA rich diets, display a better resistance to osmotic shock (Tackaert et al., 1992; Rees et al., 1994; Martins et al., 2005). In Experiment I, with the same DHA/EPA ratio, a higher dietary n-3 HUFA resulted in better resistance to osmotic shock at megalopa stage. Whereas in Experiment II, with the same total n-3 HUFA level, a higher DHA/EPA ratio resulted in better resistance to osmotic shock at the Z3, Z5 and megalopa stage. It is known that n-3 HUFA (especially DHA) are mainly incorporated in the cell membranes, and increase the permeability of membranes and hence their fluidity (Watanabe, 1993). Moreover the effect on osmoregulation may also be partially related to the modification of fatty acid composition of the gills as higher n-3 HUFA levels result in larger gill area, which in turn enhances osmo-regulatory capacity, and thus the survival upon salinity stress (Palácios et al., 2004). It should be mentioned, on

the other hand, that the capacity of osmo-regulation for mitten crab increases along with larval development (Zhao et al., 1998). Therefore the better tolerance to salinity stress in Experiment I might also be linked to the faster development of the larvae. This was however not confirmed in Experiment II, where higher DHA/EPA ratio resulted in better tolerance without showing significant growth difference of the zoeal larvae. Overall the result of the current study proved that not only higher levels of total n-3 HUFA but also the correct balance of DHA and EPA enhances larval response to stress conditions.

This study suggests that, due to its catadromous nature, *E. sinensis* larvae require the provision of preformed n-3 HUFA, with a suitable ratio of DHA to EPA in their diet. Total n-3 HUFA levels of 17 to 18 mg g^{-1} dw in both rotifers and *Artemia*, and a DHA/EPA ratio of 1.17 in rotifers and 0.56 in *Artemia* seem sufficient for optimal growth and survival. Furthermore an elevated dietary n-3 HUFA level and DHA/EPA ratio more over enhanced the tolerance of larvae to salinity shock.

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Chapter VI

General discussion

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General discussion

Despite the expansion of mitten crab aquaculture and its important role in fulfilling the growing market demand for this species for the large Chinese population, the stable production of high quality crab is still hindered by the erratic and usually low yield in the hatchery phase. As the starting point of mitten crab farming, the development of reliable hatchery techniques for high quality seed is imperative to a long-term sustainable development of mitten crab aquaculture.

Proper hatchery management ensures sufficient quantities of good quality eggs and consequently reliable offspring production. **Chapter II** revised the published and unpublished information on mitten crab hatchery techniques and laid out the current status of mitten crab larviculture in China as to inform both farmers and scientists of the techniques currently used. During the process of literature review it became clear that data and publications on a number of topics are either scarce or remain unresolved: i) Though several studies have attempted to distinguish the main geographical populations, the genetic structure of mitten crab populations within the native range remains unclear; ii) Although the importance of lipids on broodstock reproductive performance and offspring quality has been studied to some extend, quantitative requirements remain largely unknown; iii) Larval feeding regimes and nutritional requirements applied in the hatcheries are very much dependent on the farmer's experience, and little scientific research has been done in this area.

On the objective of improving sustainable mitten crab hatchery production, this thesis was conducted to better understand genetic patterns of the native mitten crab populations with a focus on aquaculture implications; to investigate the quantitative requirements of dietary phospholipids (PL) and highly unsaturated fatty acids (HUFA) during ovary maturation and its effect on reproductive performance and egg quality

using formulated feeds; and to optimize larval feeding regimes and investigate larval HUFA requirements based on the live food, rotifers and *Artemia*.

Genetic structure of the native mitten crab populations

The mitten crab is an indigenous species in China and can be found in rivers and inland lakes over an area of more than 2 million km^2, mainly in the basins of the Liaohe, Yangtze and Ou Rivers (Zhang et al., 2000). A number of studies have been conducted to distinguish these populations using biochemical and molecular makers (Wang and Yu, 1995; Gao and Zhou, 1998; Zhou and Gao, 1999); however, a conclusive pattern of geographical populations has not yet emerged due to the constraints of the methodologies applied in these studies. **Chapter III** analyzed the genetic differentiation among the native mitten crab populations using a more efficient molecular analytical tool microsatellite DNA markers. The samples were collected directly from the six main river systems, which should present the overall natural stocks of the mitten crab in China. For this study we collaborated with the Laboratory of Molecular Ecology and Fisheries Genetics of the University of Hull in the UK, where several microsatellite loci were characterized specifically from *Eriocheir sinensis* in previous studies (Hänfling and Weetman, 2003).

Our results revealed a weak population structure among the native populations, indicating that a high level of gene flow has occurred not only between geographically closer locations, but also among remote regions where a natural gene flow seems not possible. On the other hand, it is interesting to see that there was a relatively large level of differentiation within the Yangtze River samples, compared to the differentiation among river systems. Such a native population pattern is considered to be effectuated due to human mediated transportation driven by aquaculture activity and escapees from hatcheries and farms, for which the broodstock origin are often unknown. Although relocations were most frequently in the 1990s, the presumed effects of that practice are still noticeable now.

The homogeneity of the *Eriocheir sinensis* gene pool revealed in this thesis warrants aquaculturist to take sufficient care of this over-exploited species in order to avoid genetic impoverishment, and furthermore to take initiatives for genetic diversity conservation. It has been public knowledge, although with very little scientific backing, that the population of the Yangtze River contained individuals that were performing better under aquaculture conditions. The genetic study now documents that the population differentiation between the rivers has largely been lost most probably due to contamination by artificial gene flow. Hence the presumed mixed status of the mitten crab population makes it considerable more difficult to identify these individuals with a presumed better performance under aquaculture conditions. Also, assuming that a population structure has been in place before aquaculture activities started, the current lack of structure prohibits the sampling of individuals from different gene pools (river basins), which would have allowed establishing a genetically diverse broodstock which could have formed the basis for a breeding programme.

Broodstock nutrition

The life cycle of mitten crab has been closed in captivity. Owing to the fast development of the reproductive organs during maturation, high amounts of energy and nutrients need to be obtained from the exogenous feed during this process. Thus well-balanced broodstock diets are of utmost importance to improve reproductive performance and subsequently offspring quality of mitten crab.

Phospholipids (PL) constitute an important fraction of all biological membranes and play a vital role in the transport of lipids (particularly cholesterol) via hemolymph (Teshima, 1997). It also functions as a source of choline, inositol, essential fatty acids and energy, and improves diet quality as an emulsifier, and possibly as an antioxidant and feed attractant (Coutteau et al., 1997). Highly unsaturated fatty acids (HUFA), in particular ARA, EPA and DHA, are essential compounds required for cell membrane formation, osmoregulation, synthesis of prostaglandins, and they also appear to have an activating role in the immune system (Léger and Sorgeloos, 1992). Crustaceans only

have a limited ability to biosynthesize PL (Shieh, 1969; Teshima and Kanazawa, 1979; Kanazawa et al., 1985) and HUFA *de novo* (Kanazawa et al., 1979; Mourente, 1996). Thus PL and HUFA are considered essential lipid nutrients in crustacean diets. A few studies reported that PL and/or HUFA play an important role in the reproductive process of mitten crab as was described by Wen et al. (2002c) and Wu et al. (2007b). Since the catadromous nature of mitten crab, the question remains if they are required in high levels for normal ovary maturation and what are the quantitative requirements for PL and HUFA.

Two studies aiming to investigate the effects of increasing dietary PL (**Section I**) and HUFA (**Section II**) supplementation on maturation and reproductive performance of mitten crab during first ovary maturation are included in **Chapter IV**.

PL requirement

A series of isolipidic and isonitrogenous semi-purified compound diets supplemented with different levels of soybean lecithin (0, 1.2, 2.4 and 3.6%) were fed to the mitten crab broodstock in **Section I**. Our results suggested that soybean lecithin supplementation has a positive effect on ovary development (GSI), spawning percentage, egg production and fecundity of mitten crab broodstock. This confirms the function of PL in facilitating lipid transport and hence promoting ovary maturation and reproductive performance. Higher dietary PL levels resulted in higher total lipid and PL content in the eggs. From this it was speculated that eggs from females fed higher dietary PL levels would result in better larval quality as was observed for other crustaceans. The lack of significant differences in the egg hatching rate among the treatments could be explained by the constraints of *in vitro* egg incubation used in the current study, for which the egg hatching is very much influenced by hatching conditions such as suboptimal aeration and fungi infection (Bao et al., 1999).

HUFA requirement

Another series of isolipidic and isonitrogenous semi-purified compound diets supplemented with different levels of HUFA-riched fish oil (0, 0.6, 1.2, 1.8 and 2.4%) were fed to the mitten crab broodstock in **Section II**. It was shown that increased dietary HUFA levels resulted in increased HUFA levels in hepatopancreas, ovaries and eggs, but did not improve ovary maturation (GSI) and reproductive performance of mitten crab. This may indicate that normal ovary maturation does not require supplemental HUFA in the diets. However, the fact that the diets containing 1.64 and 2.07% HUFA resulted in similar HUFA content in hepatopancreas, ovary and eggs, together with the observation of earlier spawning of the females fed these diets, could indicate that a total HUFA level of 1.64% of the broodstock diet dry weight is beneficial for mitten crab.

In **Section II**, the hepatopancreas, ovary and egg HUFA levels significantly correlated with the dietary HUFA content, indicating that HUFA was efficiently transported from the broodstock diets to the tissues. Furthermore, as a lipid storage organ, the fatty acid composition of the hepatopancreas was more markedly influenced by the diet. On the other hand, ovary and eggs contained lower levels of MUFA and SFA, but higher levels of HUFA than the hepatopancreas, which may implicate the specific fatty acids requirement during ovarian development of mitten crab, though SFA and MUFA may also have been catabolised for the production of energy while PUFA and HUFA were conserved.

The ovaries and eggs generally had a lower DHA/EPA ratio (0.7-0.8) compared to the hepatopancreas (0.9-1.2). This may indicate that broodstock require more EPA than DHA during ovarian development. Thus the excess DHA may be stored in the hepatopancreas, whilst more EPA is transported to the developing ovaries. This is, however, in contrast with several other study results on marine fish and crustaceans, where DHA was preferentially incorporated into egg lipids of these species. The possible explanation could be due to catadromous nature, mitten crab possibly may not present similar fatty acid requirements as marine species, but rather present intermediate characteristics between freshwater and marine species, similar to what was reported for

freshwater prawn *Macrobrachium rosenbergii* (Cavalli et al., 1999).

Relocation of lipid stores from hepatopancreas

In crustaceans, the hepatopancreas is thought to act as the main lipid storage organ. The hepatopancreatic lipid reserves however only partially contribute to vitellogenesis and also dietary lipids must be processed rapidly through the hepatopancreas and exported to the ovaries during maturation (Harrison, 1990; Wouters et al., 2001). In nature, the main ovary maturation of mitten crab occurs during their reproductive migration from freshwater to brackish water in period of August to December (Xue et al., 1987; Li et al., 2001). Thereafter, the final maturation process merely oocyte structural changes (including yolk accumulation and lipid droplet amalgamation) with little change in biochemical composition of the ovary (Cheng et al., 1999b; Wen et al., 2001). In this chapter, the HSI, GSI, total hepatic and ovarian lipid were determined in December (after three months feeding) when the lipid mobilization from the hepatopancreas to the ovary were largely achieved. The results of both studies (**Section I** and **Section II)** demonstrated that, regardless of the treatment, both the HSI and the hepatic lipid content decreased significantly, while the GSI and the ovarian lipid content increased dramatically during maturation, but much higher than the increase of hepatopancreas. This reconfirms lipid mobilization from the hepatopancreas to the ovaries during the first ovarian maturation of mitten crab, and also that a substantial part of the lipids that accumulate in the ovaries originate from the diet, as has also been observed in the other crustaceans.

In both **Section I** and **Section II**, however, relatively high PL (8.4% of dry weight) and HUFA levels (3.5% of dry weight) were recorded in eggs from females fed diets without PL or HUFA supplementation. This may point out the importance and specific requirement for PL and HUFA in ovarian development of mitten crab. It however also demonstrates that the initial hepatic reserves before starting the feeding experiment contributed substantially to the developing ovaries, similarly as what was reported earlier by Cheng et al. (1998a) and Wen et al. (2001). If so, it would be interesting to

study nutritional requirements in broodstock animals with depleted reserves as is the case after first spawning.

Second ovary maturation

Mitten crab are capable of spawning up to three times after a single mating; however, in hatcheries, the animals are normally only used for the first spawning as survival, fecundity and egg quality drop drastically afterwards (Cheng et al., 2004; Yu et al., 2007). After the first spawning, nutrient and energy levels in the spent broodstock are depleted and need to be replenished during a period of 1-2 months, which is shorter than for the first ovarian maturation process (3-4 months). Therefore spent broodstock maybe a more appropriate model animal for nutrient requirement research because their hepatopancreatic and ovarian reserves are at their lowest level. To date, no studies have focused on second ovarian maturation. In **Section III**, a first attempt was made to study nutrient requirements during second maturation. Three natural diets (clams, sandworms and trash fish) and one artificial diet were fed to four groups of mitten crab broodstock after first spawning. The study showed that during second maturation, there is an important transfer of nutrients from the exogenous feed to the hepatopancreas, ovaries and eggs, as was observed during first maturation (**Section I** and **Section II)**, and a dramatically low survival after spawning. No significant difference in survival and reproductive performance was found among broodstock fed the formulated diet and the fresh feeds. This opens perspectives to develop nutritionally adequate formulated diets in order to improve reproductive performance during second and subsequent spawnings.

Unfortunately, from the studies reported in **Chapter IV** it was not possible to draw solid conclusions on the quantitative requirements of mitten crab broodstock for dietary PL and HUFA. The inconclusive results might have been caused by the high variability between individual females. Broodstock studies require large facilities. Moreover mitten crabs are very aggressive and need to be kept at low density (less than 5 crab m^{-2}). Due to the limitations of the hatchery where the studies were performed, it was therefore practically impossible to further increase the number of replicates or the number of

animals per replicate. In addition, over the long rearing period (9-10 months), high mortality also could not be avoided (data not shown in the technical papers). Though broodstock origin was carefully selected and animals of similar size were used, their nutritional and genetic background remained unknown. As we have demonstrated in **Chapter III**, the low genetic diversity among natural stocks and the high inbreeding potential in hatcheries might have resulted in inferior and variable crab quality, and consequently high variability among the replicates in the experiments.

Larval feeding regimes

Like other brachyuran species, mitten crab Z1 larvae hatch with little or no yolk reserve and almost start eating exogenous feed immediately after hatching. Moreover, the pelagic zoea larvae are rather small at hatch, but they grow fast and almost double their body weight after each molt or metamorphosis. Thus supply of a readily available and digestible feed is therefore crucially important for the larvae. Rotifers and *Artemia* are, for the time being, the most practical live food used in the hatcheries due to their convenience in use and availability.

In assessing a diet in terms of its success or failure in sustaining zoea development, two factors should be considered: can the prey be caught and ingested; once ingested, does the prey provide the necessary nutritive value (Sulkin, 1975). A primary study investigating *Artemia* ingestion with or without rotifers as co-feed was performed on individual larvae at each zoea stage **(Chapter V, Section I).** Mitten crab Z1 larvae proved capable of catching instar-1 *Artemia* nauplii, however it seemed they were difficult to completely ingest. In contrast, rotifers were preferably ingested at the Z1 and Z2 stage due to their smaller size and slower swimming behavior. It was furthermore suggested that *Artemia* should be introduced to the rearing system from Z3 onwards, not only due to their bigger size (and hence better feeding efficiency of the larvae), but also their better nutritional value. This is in agreement with observations in other brachyuran larvae. Moreover, the study showed that prey consumption increased not only with larval development but was also significantly influenced by *Artemia* density.

Based on the observation on individual larvae in **Section I**, the optimal time to wean larvae from rotifers onto *Artemia* (**Experiment I**) and the optimal rotifer and *Artemia* feeding density (**Experiment II** and **III**) were further clarified in a series of communal larval rearing experiments as described in **Chapter V, Section II**. **Experiment I** confirmed the results from **Section I** and further stressed that rotifers are the best live feed for mitten crab larvae at early stages (Z1-Z2), whereas *Artemia* appear to be better than rotifers for later stages. This is also in line with practical considerations because it limits the dependence on rotifer cultures, which are subject to unpredictable crashes (Rombaut, 2001). However, introducing *Artemia* nauplii too early is also not ideal. Even when starved, *Artemia* nauplii will molt into second instar metanauplii which are sometimes larger than the larvae. Moreover in commercial operations, it may also be more cost effective to delay the introduction of *Artemia* for as long as possible due to the high cost of the cysts (Davis, 2003).

The results of **Experiment II** and **III** suggested that, under the experimental conditions, the optimal rotifer feeding density for Z1 and Z2 was 15 and 20 mL^{-1}, respectively; while the optimal *Artemia* feeding density for Z3, Z4 and Z5 was 3, 5 and 8 mL^{-1}, respectively. It should however be mentioned that, in practice, the optimal feeding regime should take into account performance of the larvae as well as feed cost.

Larval nutrition

Besides suboptimal feeding regimes, nutritional non-balanced feeds may also contribute to the highly variable survival and indirectly increase susceptibility to diseases, which are often encountered in the hatchery, particularly when larvae are under stressful condition. From a nutritional point of view, rotifers and *Artemia* are not ideal diets for marine crustacean larvae because they naturally lack n-3 HUFA, but instead are rich in linolenic acid (18:3 n-3) and to a lesser extent linoleic acid (18:2 n-6) (Sargent et al., 1999). Most marine crustaceans, especially young stages, such as larvae and juveniles, have a limited ability to synthesize HUFA *de novo* (Suprayudi et al., 2004).

A process known as bioencapsulation enables to improve the nutritional quality of rotifers and *Artemia* by enriching them with n-3 HUFA rich products, which provides an excellent tool to investigate larval nutritional requirement via live food (Merchie et al., 1995; Velu and Munuswamy, 2004). In **Chapter V, Section III**, the effect of varying levels of dietary n-3 HUFA and DHA/EPA ratios on growth, survival and osmotic stress tolerance of mitten crab larvae was studied. The rotifers and *Artemia* were enriched with ICES fish oil emulsions, which contained n-3 HUFA levels of 0, 30 and 50%, but with the same DHA/EPA ratio of 0.6 (**Experiment I**); or the same n-3 HUFA level (30%), but different DHA/EPA ratios (0.6, 2 and 4) (**Experiment II**).

Low dietary n-3 HUFA levels resulted in inferior growth in terms of individual body weight and larval development (LSI); and a significantly inferior larval development and lower megalopa survival were obtained with lower dietary DHA/EPA ratio. In addition, the offspring quality was evaluated using the salinity stress test. Higher dietary HUFA level and DHA/EPA ratio resulted in better tolerance to a salinity shock. The high larval survival upon osmotic shock is probably related to the enhanced osmo-regulatory capacity of the cells due to the higher n-3 HUFA levels in the cell membranes.

Overall, from the results in **Chapter V**, **Section III** a total n-3 HUFA level of 17 to 18 mg g^{-1} dw in both rotifers and *Artemia*, with a DHA/EPA ratio of 1.17 in rotifers and 0.56 in *Artemia* are recommended for optimal growth and survival of mitten crab larvae, which is similar to what was found for most marine crustacean species.

General conclusions

This thesis, for the first time, has documented the population structure of natural mitten crab stocks using more efficient microsatellite DNA markers, and relates the weak genetic structure to past and present mitten crab aquaculture practices. The importance of genetic diversity conservation of this over-exploited species is stressed. Lack of genetic structure, presumably caused by aquaculture activities, also constitutes to some

extent, a mixed opportunity for breeding.

It furthermore showed the positive effects of dietary PL on maturation and reproductive performance, meanwhile demonstrated the mobilization and transport of HUFA between the different tissues during the maturation process. The knowledge and analytical information provided in this thesis are considered a good starting point to better understand the nutritional requirements of mitten crab broodstock and to formulate effective and nutritionally balanced artificial diets.

Finally, considerable progress was made in developing optimal larval feeding regimes and in improving the live feed composition through investigating requirements for dietary n-3 HUFA level and DHA/EPA ratio.

Suggestions for further research

- Further studies should clarify the optimal PL and HUFA level in mitten crab broodstock diets, not only in terms of reproductive performance, but also in terms of offspring quality. Based on the research results, the development of effective broodstock diets should be a priority.

- The nutrient requirements of male breeders for optimal spermatophore development and high sperm quality should receive special attention, as most publications focus on female breeders only.

- Optimized feeding regime should be further verified in a large-scale experiment and also in a commercial setting.

- As for many other intensive aquaculture operations, another important challenge for crab larval rearing is the development of safe and sustainable prophylactic treatments to prevent microbial interactions. The last decade, antibiotics have been widely used in mitten crab larviculture to prevent disease outbreaks. Nowadays however its application

is increasingly restricted because of environmental and food safety considerations; hence, the investigation into alternative solutions is needed. Probiotics are potentially useful tools in disease prevention and control through i) balancing the microflora and reducing the pathogenic bacterial load in the culture water; ii) providing essential nutrients and digestive enzymes to enhance the nutritional status of the cultured animals; iii) direct uptake or decomposition of organic material or toxic substances present in the water. In an earlier study, we tested a commercial probiotic product developed for shrimp and occasionally obtained better survival compared to a control. The result was however not constant. More attention should be paid to develop specific probiotics for mitten crab.

- Efforts should also be directed towards genetic selection for improved growth, adaptability to extreme culture conditions and disease resistance.

Chapter VII

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Summary

Mitten crab is one of the few crustacean species that is being produced on an industrial scale. For Chinese people it is considered a luxury aquatic food due to its delicate flavor and high nutritional quality and hence fetches high market prices. Although breakthroughs in seed production and larval rearing techniques of mitten crab have been achieved in the early 1980s, a fast expansion of hatchery production only took of in the 1990s, with the dramatic decline in natural recruitment and the collapse of penaeid shrimp culture in China.

The main obstacle for mitten crab hatchery production is the variable and usually low larval survival, which is believed to relate to poor broodstock management and suboptimal larval rearing techniques. The main research scope of this thesis was to clarify the genetic structure of natural mitten crab stocks in order to select broodstock with known genetic origin (e.g. better reproductive, growth patterns and disease resistance); to improve reproductive performance, egg and offspring quality through enhancing nutritional status of broodstock; and to optimize larval feeding regimes and nutrition. Research on the above issues should lead to improvement of mitten crab hatchery techniques, and thus enhance and sustain mitten crab hatchery production (**Chapter I**).

Mitten crab hatchery production and farming are being performed almost exclusively in China. Many techniques have only been published as brief descriptions of local farmers' experience and most of these articles were written in Chinese. This is probably why mitten crab research has only attracted very little interest of the international scientific community. **Chapter II** provides general information of natural stock distribution, biological characteristics and behavior of mitten crab; reviews the hatchery techniques in aspects of broodstock maturation, spawning and larval rearing and points out the main bottlenecks of current mitten crab hatchery operations. The information collected

in this chapter is not only providing background knowledge for the research subjects of this thesis, but also giving a general overview of mitten crab aquaculture to researchers and farmers worldwide.

A better understanding of the population genetic structure is crucial for the control of the exploitation and the enhancement of natural stock conservation of an aquaculture species. **Chapter III** investigated the genetic composition of native mitten crab populations by screening samples from nine locations covering six river systems at six microsatellite loci. Genetic diversity (allelic richness, and observed and expected heterozygosity) and global and pairwise *F*st were used to determine the genetic structure among the populations. The results showed less structuring among the native populations, indicating high gene flow occurred not only between closer locations, but also between geographically remote river systems. The frequent exchange of broodstock, juvenile and larvae from different origins and escapees from hatcheries, culture ponds and net enclosures are considered as main causes and therefore conservation polices such as genetic diversity protection of localized populations and cessation of human-facilitated introduction were emphasized in this chapter. In addition, **Chapter III** also integrated the current data with those reported by Herborg et al. (2007) on three invasive mitten crab populations from Europe and America, and one native sample from Liaohe. It further confirmed that a founder effect and a stepping stone colonization from China to Europe to North America were obvious in the invasive populations as observed by a gradually decrease in expected heterozygosity and a reduced allelic richness, compared to the native populations.

Broodstock management is the first concern of any successful aquaculture operation, as it influences egg and larval quality. Poor hatchery performance may, in part, be related to nutritional imbalances of the broodstock. Phospholipids and HUFA are considered as important nutrients for crustacean broodstock maturation. However the quantitative requirements for both nutrients have not yet been established for mitten crab broodstock. **Chapter IV** investigated the effects of increasing dietary soybean lecithin and HUFA supplementation on ovary maturation, fecundity, egg production and egg quality of

mitten crab.

In **Section I**, four semi-purified formulated diets supplemented with 0% (Diet 1), 1.2% (Diet 2), 2.4% (Diet 3) and 3.6% (Diet 4) soybean lecithin were fed to the female crabs for a period of 7 months. Male crabs were introduced into the female rearing system in March, and mating, spawning and egg hatching occurred within the following two months. The result showed that the females fed Diet 3 had a significantly higher gonad somatic index (GSI) than the females fed Diet 1, while the females fed Diet 2 had a significantly higher hepatic moisture content and lower hepatic lipid content than the other groups; the females fed Diet 4 and Diet 3 had higher spawning rate (95% and 92%, respectively) compared to the females fed Diet 2 and Diet 1 (both 81%). Furthermore egg production and fecundity increased with increasing dietary soybean lecithin level, with females fed Diet 1 and Diet 2 having significantly lower values than the females fed Diet 4. It was concluded that dietary soybean lecithin supplementation has a positive effect on ovary development and reproductive performance of mitten crab broodstock.

In **Section II**, five isolipidic and isonitrogenous diets were formulated to contain different HUFA levels (0.14, 0.65, 1.15, 1.64 and 2.07%) by the supplementation of HUFA-rich fish oil and fed to the female crabs for a period of 8 months. A similar protocol was applied as being described in Section I. There were no significant differences in GSI, HSI, total ovarian and hepatic lipid content between the treatments. The reproductive performance was also similar, except that females fed the diets containing higher HUFA levels tended to spawn earlier. A significant positive correlation was observed between the hepatopancreas, ovary and egg HUFA levels and the dietary HUFA content; but no significant correlation could be found between muscle HUFA level and dietary HUFA content. The fact that the diets containing 1.64 and 2.07 % HUFA resulted in similar HUFA levels in the hepatopancreas, ovary and eggs, could indicate that a total HUFA level of 1.64% of the diet dry weight is required for mitten crab broodstock diets. The fatty acid mobilization from the hepatopancreas to the ovary and eggs are also discussed in this section.

With the purpose of extending the reproduction window, but also finding a more sensitive model to be able to more accurately investigate broodstock nutritional requirements, a preliminary experiment was setup using spent spawners (**Section III).** Three natural diets (clams, sandworms and trash fish) and one artificial diet were fed to re-maturing broodstock after first spawning. The HUFA profile of the eggs, ovary and hepatopancreas reflected that of the experimental diets, indicating an important lipid transfer from the exogenous feed to these tissues during secondary ovary maturation. Except the higher survival of starved Z1 larvae originating from crabs fed trash fish, and the bigger egg diameter from crabs fed clams, no significant differences between treatments were found for any of the other parameters (crab weight, survival and relative fecundity).

Many factors affect the survival of cultured larvae. One of the most important factors is feeding and nutrition. Rotifers and *Artemia* nauplii are the most commonly used live food in mitten crab hatcheries. Feeding practices in hatcheries depend largely on the experience of the farmers and very little research has been performed. **Chapter V** investigated the feeding regimes (**Section I** and **II**) and the HUFA requirements (**Section III**) of mitten crab larvae through a series of experiments.

In **Section I,** the ingestion rate of newly-hatched *Artemia* nauplii by individually kept zoea larvae was studied. At each zoeal stage, 10 groups of 10 larvae were reared individually in glass beakers and fed with increasing densities of newly-hatched *Artemia* nauplii (0.5, 2.5, 5, 10 and 20 ind mL^{-1}) with or without rotifers (*Brachionus rotundiformis*, 15-25 ind mL^{-1}) as a co-feed. The average number of ingested *Artemia* over 24 h was measured and the average larval development (Larval Stage Index, LSI) was compared. Although Z1 larvae were able to consume *Artemia* nauplii, it was recommended that rotifers are better offered to Z1-Z2 stages. *Artemia* can be introduced into the rearing system at a density of 0.5 ind mL^{-1} from Z3 onwards with or without rotifers as co-feed. An *Artemia* density of 5 ind mL^{-1} should be provided to Z4-Z5 stages.

In **Section II**, further experiments were set up using communal larval rearing systems to assess the optimal timing of introducing *Artemia* into the diet (Experiment I), and to investigate the optimal rotifer feeding density at the early larval stages (Z1-Z2) (Experiment II) and optimum *Artemia* feeding density at later zoeal stages (Z3-Z5) (Experiment III). Six replicates per treatment were performed using 2 L plastic beakers containing 1.5 L diluted seawater (20 g L^{-1}). The beakers were stocked with 150 Z1 in Experiment I, 200 Z1 in Experiment II and 150 Z3 in Experiment III. Different combinations and densities of rotifers and newly-hatched *Artemia* nauplii were fed to zoea larvae. Average survival at each stage, LSI, duration of each zoeal stage and individual megalopa weight were compared among treatments. The results confirmed the previous observation that rotifers are the best live feed for Z1-Z2 larvae and *Artemia* appear to be better than rotifers for later stages. The optimal rotifer feeding density for Z1 and Z2 was 15 and 20 mL^{-1}, respectively; while the optimal *Artemia* feeding density for Z3, Z4 and Z5 was 3, 5 and 8 mL^{-1}, respectively.

In **Section III**, the effect of varying levels of dietary n-3 HUFA and DHA/EPA ratios on growth, survival and osmotic stress tolerance of mitten crab zoea larvae was studied in two separate experiments. In experiment I, larvae were fed rotifers and *Artemia* enriched with ICES emulsions with 0, 30 and 50 % total n-3 HUFA levels but with the same DHA/EPA ratio of 0.6. In experiment II, larvae were fed different combinations of enriched rotifers and *Artemia*, in which, rotifers were enriched with emulsions containing 30% total n-3 HUFA, but different DHA/EPA ratio of 0.6, 2 and 4; while *Artemia* were enriched with emulsions containing 30% n-3 HUFA, but a DHA/EPA ratio of either 0.6 or 4. In both experiments, un-enriched rotifers cultured on baker's yeast and newly-hatched *Artemia* nauplii were used as control diets. Larvae were fed rotifers at Z1-Z2 stage; upon reaching Z3 stage, *Artemia* was introduced. The overall results concluded that total n-3 HUFA levels of 17 to 18 mg g^{-1} dw in both rotifers and *Artemia*, and a DHA/EPA ratio of 1.17 in rotifers and 0.56 in *Artemia* were sufficient for optimal growth and survival. An elevated dietary n-3 HUFA level and DHA/EPA ratio however enhanced the tolerance of larvae to salinity shock.

The knowledge obtained through this thesis work should contribute towards the development of improved hatchery techniques for mitten crab in aspect of genetic diversity conservation of natural stocks, better understanding of the nutrient requirements and metabolism of mitten crab broodstock and the development of optimal larval rearing protocols.

Samenvatting

De wolhandkrab is een van de weinige soorten crustaceeën die op een industriële schaal gekweekt wordt. Chinezen beschouwen het als een luxevoedsel wegens de delicate smaak en de hoge nutritionele waarde en bijgevolg zijn de marktprijzen hoog. Hoewel er reeds een doorbraak in de productie van juvenielen en kweektechnieken voor larven gerealiseerd werd in de jaren 1980, duurde het tot de jaren 1990, door de dramatische daling van de natuurlijke rekrutering en het ineenstorten van de garnalenkweek in China, vooraleer een snelle expansie van productie in broedhuizen zich voltrok.

Het grootste probleem bij de broedhuisfase van de wolhandkrab is de variabele en gewoonlijk lage overleving van de larven, welke geacht wordt gerelateerd te zijn aan slecht management van de ouderdieren en suboptimale larvale kweektechnieken. De belangrijkste onderzoeksdoelstellingen van deze thesis waren de genetische struktuur van de natuurlijke wolhandkrabpopulaties uit te klaren om zo ouderdieren met een gekende genetische achtergrond te kunnen kiezen (bijv. met betere voortplantings- en groeikenmerken en ziekteresistentie); de voortplanting en kwaliteit van de eieren en nakomlingen te verbeteren door middel van het verbeteren van de nutritionele status van de ouderdieren; en de voeding van de larven te verbeteren. Onderzoek naar deze aspecten zou moeten leiden naar een verbetering van de broedhuistechnieken voor de wolhandkrab en dus de productie in broedhuizen verbeteren en ondersteunen (**Hoofdstuk I**).

De productie van juvenielen en vetmesterij van wolhandkrab wordt bijna exclusief in China uitgevoerd. Veel technieken werden enkel beknopt beschreven als de ervaringen van lokale kwekers en de meeste van deze artiekels werden in het Chinees geschreven. Dit is waarschijnlijk de reden waarom onderzoek op de wolhandkrab slechts heel weinig aandacht gekregen heeft van de internationale onderzoeksgemeenschap. **Hoofdstuk II** verschaft algemene informatie over de verspreiding van de natuurlijke populaties,

biologische kenmerken en gedrag van wolhandkrabben; en geeft een overzicht van de broedhuistechnieken met betrekking tot maturatie van de ouderdieren, ei-afleg en larvale kweek en het geeft de belangrijkste huidige knelpunten aan van broedhuizen. De informatie die in dit hoofdstuk verzameld werd, dient niet alleen als achtergrondinformatie voor de onderzoeksonderwerpen van deze thesis, maar geven ook een algemeen overzicht van de aquakultuur van de wolhandkrab voor zowel onderzoekers als kwekers.

Een beter kennis van de genetische struktuur van de populaties is cruciaal voor de controle van de exploitatie en het bevorderen van de bescherming van de natuurlijke stocks van een aquakultuursoort. **Hoofdstuk III** onderzocht de genetische samenstelling van de inheemse wolhandkrabpopulaties door het onderzoeken van stalen van negen locaties over zes riviersystemen met behulp van zes microsatelliet loci. Genetische diversiteit (allele rijkdom en waargenomen en verwachte heterozygositeit) en globale en gepaarde *F*st werden gebruikt om de genetische struktuur van de populaties te bepalen. De resultaten toonden weinig structurering bij de inheemse populaties, hetgeen een hoge uitwisseling van genen aangeeft, niet enkel tussen dichtbijgelegen locaties, maar ook tussen geografisch verafgelegen riviersystemen. De veelvuldige uitwisseling van ouderdieren en larven van verschillende oorsprong en ontsnappingen uit broedhuizen en kweekvijvers en netkooien worden beschouwd als de belangrijkste oorzaken hiervan en daarom werden de noodzaak voor beschermingsmaatregelen zoals het behoud van de genetische diversiteit van lokale populaties en het stopzetten van menselijke introducties benadrukt in dit hoofdstuk. Daarenboven, werden in **Hoofdstuk III** de huidige data ook samengevoegd met deze van Herborg et al. (2007) van 3 invasieve wolhandkrabpopulaties van Europa en Amerika, en 1 inheems staal van Liaohe. Dit bevestigde verder het “founder effect” en een “stepping stone” kolonizatie van China naar Europa naar Noord-Amerika in de invasieve populaties zoals vastgesteld door een geleidelijke afname in de verwachte heterozygositeit en de verminderde allele rijkdom, in vergelijking met de inheemse populaties.

Het mangement van de ouderdieren is de eerste bekommernis van elke

aquakultuuractiviteit, aangezien het een invloed heeft op de kwaliteit van de eieren en de larven. Slechte resultaten in een broedhuis kunnen, ten dele, te wijten zijn aan nutrionele onevenwichten bij de ouderdieren. Phospholipiden en HUFA worden beschouwd als belangrijke nutriënten voor de maturatie van ouderdieren van schaaldieren. De kwantitatieve vereisten voor beide nutrienten werden echter nog niet vastgelegd voor ouderdieren van de wolhandkrab. **Hoofdstuk IV** onderzocht het effect van supplementatie met soyalecithine en HUFA in het voeder op maturatie van de ovaria, de fecunditeit en eiproductie en eikwaliteit van de wolhandkrab.

In **Deel I**, werden vier semi-gepurifieerde voeders, gesupplementeerd met 0% (voeder 1), 1.2% (voeder 2), 2.4% (voeder 3) en 3.6% (voeder 4) soyalecithine, gevoederd aan vrouwelijke krabben voor een periode van 7 maanden. Mannelijke krabben werden geintroduceerd in het kweeksysteem in maart, en bevruchting, afleg en ontluiken van de eieren gebeurde in de daaropvolgende twee maanden. De resultaten toonden aan dat vrouwtjes die gevoederd werden met voeder 3 een significant hogere gonadosomatische index (GSI) hadden dan vrouwtjes gevoederd met voeder 1, terwijl de dieren gevoederd met voeder 2 een significant hoger vochtgehalte en een significant lager vetgehalte in de hepatopancreas hadden; vrouwtjes gevoederd met voeder 4 en voeder 3 hadden een hogere ei-afleg (95% en 92%, respectievelijk) in vergelijking met vrouwtjes gevoederd met voeder 2 en voeder 1 (beide 81%). Verder namen eiproductie en fecunditeit toe met toenemend gehalte soyalecithine in het voeder, waarbij vrouwtjes gevoederd met voeder 1 en 2 significant lagere waarden hadden dan vrouwtjes gevoederd met voeder 4. Er werd besloten dat supplementatie van soyalecithine in het voeder een positief effect heeft op de ontwikkeling van de ovaria en het succes van de voortplanting van de wolhandkrab.

In **Deel II**, werden vijf isolipidische en isonitrogene voeders geformuleerd met verschillende gehaltes aan HUFA (0.14, 0.65, 1.15, 1.64 and 2.07%) door middel van supplementatie met HUFA-rijke visolie en gevoederd aan vrouwelijke krabben voor 8 maanden. Een gelijkaardig protocol als dat beschreven in deel I werd gebruikt. Er waren geen significante verschillen in GSI, HSI en totaal gehalte aan lipiden van de

hepatopancreas en de ovaria tussen de verschillende behandelingen. Voortplantingssucces was ook gelijkaardig, behalve dat dieren die gevoederd werden met voeders met hogere gehaltes aan HUFA de neiging hadden eerder af te leggen. Een significante positieve correlatie werd vastgesteld tussen het gehalte aan HUFA in de hepatopancreas, ovaria en eieren en het HUFA gehalte in het voeder; maar er werd geen significante correlatie gevonden tussen het HUFA gehalte in de spieren en deze in het voeder. Het feit dat de voeders met 1.64% en 2.07% HUFA resulteerden in gelijkaardige HUFA gehaltes in de hepatopancreas, ovaria en eieren zou er kunnen op wijzen dat een totaal HUFA gehalte van 1.64% van het voeder droog gewicht nodig is voor voeders voor ouderdieren van de wolhandkrab. De mobilizatie van vetzuren van de hepatopancreas naar de ovaria en eieren wordt ook besproken in dit deel.

Met het doel om de reproductieperiode te verlengen, maar ook om een meer gevoelig model te vinden om nutritionele behoeften van ouderdieren meer accuraat te kunnen onderzoeken, werd een preliminaire test opgezet met ouderdieren die reeds eitjes afgelegd hebben (**Deel III**). Drie natuurlijke voeders (venusschelpen, polychaeten en kleine visjes) en een artificieel voeder werd gevoederd aan ouderdieren na de eerste ei-afleg tijdens de rematuratie. Het HUFA profiel van de eieren, ovaria en hepatopancreas weerspiegelden dat van de experimentele voeders, hetgeen aangaf dat er een belangrijke transfer van lipiden van het voeder naar deze weefsels plaatsvond tijdens de tweede maturatie van de ovaria. Behalve de hogere overleving van gestarveerde Z1 larven voortgebracht door de krabben die vis gevoederd werden, en de grotere eidiameter van krabben gevoederd met venusschelpen, werden geen significante verschillen vastgesteld tussen de behandelingen voor de overige parameters (gewicht en overleving van de krabben en relatieve fecunditeit).

Veel factoren beinvloeden de overleving van gekweekte larven. Een van de belangrijkste factoren is voeding en nutritie. Rotiferen en *Artemia* zijn de meest gebruikte levende voedsels in broedhuizen voor wolhandkrabben. Voedertechnieken in broedhuizen hangen voor een groot deel af van de ervaring van de kwekers en erg weinig onderzoek werd uitgevoerd. **Hoofdstuk V** onderzocht het voederregime (**Deel I**

and II) en de vereisten aan HUFA (**Deel III**) van de larven van de wolhandkrab in een reeks experimenten.

In Deel I, werd de opname van vers-ontloken *Artemia* nauplii door individueel gehuisveste zoea larven bestudeerd. Voor elk zoea stadium, werden 10 groepen van 10 larven individueel gehuisvest in glazen bekers en gevoed met toenemende densiteiten van pas-ontloken *Artemia* nauplii(0.5, 2.5, 5, 10 and 20 ind. mL^{-1}) met of zonder rotiferen (*Brachionus rotundiformis*, 15-25 ind. mL^{-1}) als extra voedsel. Het gemiddeld aantal opgenomen *Artemia* over 24 uur werd gemeten en de gemiddelde larvale ontwikkeling (Larvale Stadium Index, LSI) werd vergeleken. Hoewel Z1 larven in staat waren om *Artemia* nauplii te consumeren, werd aanbevolen dat er beter rotiferen gegeven worden aan Z 1-Z2 stadia. *Artemia* kan geintroduceerd worden in het kweeksysteem aan een densiteit van 0.5 ind. mL^{-1} vanaf Z3 met of zonder toevoeging van rotiferen. Een *Artemia* densiteit van 5 ind. mL^{-1} moet voorzien worden in het Z4-Z5 stadium.

In **Deel II**, werden verdere experimenten opgezet met gemeenschappelijke larvale kweeksystemen om het optimale tijdstip voor introductie van *Artemia* in het dieet (Experiment I), de optimale densiteit aan rotiferen voor de vroege larvale stadia (Z1-Z2) (Experiment II) en de optimale densiteit aan *Artemia* voor de latere zoea stadia (Z3-Z5) (Experiment 3) te bepalen. Er werden zes herhaling per behandeling uitgevoerd in 2 L plastiek bekers met 1.5 L brak water (20 g L^{-1}). De bekers werden gestockeerd met 150 Z1 in experiment I, 200 Z1 in experiment II and 150 Z3 in experiment III. Verschillende combinaties en densiteiten van rotiferen en pas-ontloken *Artemia* nauplii werden gevoederd aan de zoea larven. De gemiddelde overleving bij elk stadium, LSI, de duur van elk larvaal stadium en het individueel megalopa gewicht werden vergeleken tussen de behandelingen. De resultaten bevestigden eerdere observaties dat rotiferen het beste levend voedsel zijn voor Z1-Z2 larven en dat *Artemia* beter blijkt te zijn dan rotiferen voor de latere stadia. De optimale rotiferen densiteit voor Z1 en Z2 was 15 and 20 mL^{-1}, respectievelijk; terwijl de optimale *Artemia* densiteit voor Z3, Z4 en Z5 6, 10 en 15 mL^{-1}, respectievelijk was.

In **Deel III**, werd in twee afzonderlijke proeven, het effect bestudeerd van verschillende n-3 HUFA gehaltes en DHA/EPA verhoudingen in het voeder op groei, overleving en tolerantie tegen osmotische stress van larven van de wolhandkrab. In experiment I, werden de larven gevoed met rotiferen en *Artemia* aangerijkt met ICES emulsies met 0, 30 and 50 % totaal n-3 HUFA gehalte, maar met dezelfde DHA/EPA verhouding van 0.6. In experiment II, werden de larven gevoed met verschillende combinaties van aangerijkte rotiferen en *Artemia*, waarbij de rotiferen aangerijkt werden met emulsies met 30% totaal n-3 HUFA, maar verschillende DHA/EPA verhouding van 0.6, 2 en 4; terwijl Artemia aangerijkt werd met emulsies met 30% n-3 HUFA, maar met een DHA/EPA verhouding van ofwel 0.6 ofwel 4. In beide experimenten werden niet-aangerijkte rotiferen, gekweekt op bakkersgist, en pas-ontloken *Artemia* nauplii gebruikt als controle voeder. De larven werden gevoed met rotiferen in het Z1-Z2 stadium, en wanneer het Z3 stadium bereikt werd met Artemia. Uit de algemene resultaten werd besloten dat een total n-3 HUFA gehalte van 17 tot 18 mg g^{-1} dw in zowel rotiferen als *Artemia*, en een DHA/EPA verhouding van 1.17 in rotiferen en 0.56 in *Artemia* voldoende zijn voor een optimale groei en overleving. Een hoger n-3 HUFA gehalte en DHA/EPA verhouding echter bevorderde de tolerantie van de larven tegen saliniteitsstress.

De kennis opgedaan tijdens deze thesisstudie zou moeten bijdragen tot de ontwikkeling van verbeterde broedhuistechnieken voor de wolhandkrab op het gebied van bescherming van de genetische diversiteit van de natuurlijke populaties, een beter inzicht in de vereisten aan nutrienten en het metabolisme hiervan en de ontwikkeling van optimale protocols voor de larvale kweek.

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Oct. – Dec. 2001 Characterization of *Artemia* strains from Tibet, China. Laboratory of Aquaculture & Artemia Reference Centre, Gent University (INCO project)

Sept. – Dec. 2000 Characterization of *Artemia* strains from Tibet, China. Laboratory of Aquaculture & Artemia Reference Centre, Gent University (INCO project)

Apr. – Jul. 1994 *Artemia* nutrition study. Laboratory of Aquaculture & Artemia Reference Centre, Gent University (VLIR project)

Local involvement (Salt Research Institute of China National Salt Industry Corporation)

2001 – 2004 Technical support for local INVE companies (mainly on *Artemia* biomass production, part-time)

2001 – 2003 High density rotifer culture in re-circulation system, *Artemia* biomass enrichment (National research project) / Characterization of *Artemia* strains in China (INCO project)

1996 – 1998 Use of artificial diets based on *Artemia* biomass in Chinese mitten crab *Eriocheir sinensis* larviculture (National research project, coordinator)

1994 – 1996 High-density rotifer culture and production of rotifer resting eggs (National research project)

1992 – 1994 Characterization of *Artemia* strains in China / shrimp and crab larviculture (EEC/VLIR project)

1991 – 1992 Utilization of polysaccharides and protein of microalgae *Dunaliella salina* in the human food (National research project)

1989 – 1991 Extraction of β-carotene from microalgae *Dunaliella salina* and its application in human food (UNDP project)

International conference and workshop participation

Sept. 2005 Larvi'05, Fourth Fish and Shellfish Larviculture Symposium. European Aquaculture Society, Gent University, Belgium

Dec. 2002 Workshop on Crab Larviculture, Gent University, Belgium

Apr. 2002 World Aquaculture Society Conference, Beijing, China

Oct. 2002 Workshop on *Artemia* Biodiversity (INCO), Beijing, China

Sept. 2001 Larvi'01, Third Fish and Shellfish Larviculture Symposium. European Aquaculture Society, Gent University, Belgium

Nov. 2000 Workshop of the FWO working group: Nutritional & Microbial Studies in larval aquaculture, The University of Namur, Belgium

Oct. 1996 Fourth Asian Fisheries Forum, Beijing, China

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