Investigation of the aquaculture potential of an Australian freshwater prawn, *Macrobrachium spinipes* (Schenkel, 1902), with emphasis on spawning induction, larval and nursery culture

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STATEMENT ON THE CONTRIBUTION OF OTHERS

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Declaration on Ethics

This research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition, 2004 and the Qld Animal Care and Protection Act, 2001. The proposed research study received animal ethics approval from the JCU Animal ethics Committee Approval Number # A1046

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Abstract

Modern aquaculture of freshwater prawns began in 1960's after the life cycle of *Macrobrachium rosenbergii* was first closed. The industry has since expanded rapidly with global annual production reaching 443,959 tonnes in 2009. The Australian native freshwater prawn species formerly known as *M. rosenbergii* was recently renamed as *Macrobrachium spinipes*. The past attempts to commercially culture the native Australian freshwater prawn has never taken off citing various problems. In view that new molecular evidence showed native Australian freshwater prawn encompasses four lineages, this study investigated broodstock management, larval and nursery culture techniques for the lineage II *M. spinipes* sourced from tropical north Queensland.

Spawn induction of *M. spinipes* outside its natural breeding season (November to March) was investigated by temperature and photoperiod manipulation. Mature females (15) were kept individually under controlled water temperatures of 24, 27 and 30 °C with photoperiod adjusted to 14.5 h light: 9.5 h dark while the control was under ambient conditions. The experiment ran from May to August for 84 days and throughout the experimental duration, no spawning was recorded in any treatments. Despite ambient temperature dropping to as low as 12 °C for the control, no mortality was recorded in all treatments except a single death found for 30 °C treatment and common moults occurred in all treatments even during the coldest period in the control. Hence *M. spinipes* showed high tolerance to low water temperature and potential for grow-out culture under cooler climates.

The effects of microalgae *Nannochloropsis* sp. addition and concentration ('green water') on larval survival, development and growth of newly hatched larvae of *M. spinipes* were investigated under the condition of no algae addition ('clear water') and four *Nannochloropsis* concentrations of 2.5, 6.25, 12.5 and 25 x 10^5 cells ml^{-1}. Survival to PL at the two higher algae concentrations of 12.5 and 25 x 10^5 cells ml^{-1} (70.8 % and 63.3 %, respectively) were significantly higher ($P<0.05$) than the two lower concentrations and the 'clear water' treatments (26.7 %, 35.0 % and 30.0 %), respectively. Meanwhile, the fastest mean development to PL (30.6 days) was obtained at the highest algal density, which was 14 days shorter than that of the 'clear water' treatment (44.3 days). Mean dry weights of newly settled PL's in the two high algal density treatments were also significantly heavier ($P<0.05$) than in the lowest algal density and the 'clear water' treatments. The results clearly showed that in comparison with the 'clear water' method, 'green water' culture by adding *Nannochloropsis* sp. potentially could substantially improve larval performance of *M. spinipes,* however such beneficial effects only became significant when the concentration of algae added reached a threshold level.

A further experiment was conducted to evaluate the effects of addition of higher microalgae concentrations as compared to *Artemia* enrichment on larval growth, survival and fatty acid composition of *M. spinipes* larvae and their interactions. The newly hatched larvae were cultured with no addition of algae ('clear water') and *Nannochloropsis* sp. added ('green water') at two concentrations of 2.5 (low) and 10 x 10^6 cells ml^{-1}(high) and fed with either enriched or non-enriched *Artemia:* i.e.1) 'clearwater' + enriched *Artemia*; 2) 'clearwater' + non-enriched *Artemia*; 3) 2.5 x 10^6 cells ml^{-1} + enriched *Artemia;* 4) 2.5 x 10^6 cells ml^{-1} + non-enriched *Artemia*; 5) 10 x10^6 cells ml^{-1} + enriched *Artemia* and 6) 10 x10^6 cells ml^{-1} +

non-enriched *Artemia*. The results showed that under 'clear water' culture, larvae fed non-enriched *Artemia* resulted in total mortality while *Artemia* enrichment improved survival to 18 %; at the low algal density ($2.5x10^6$ cells ml^{-1}), larval survival also improved with *Artemia* enrichment (55.8 vs. 80 %). However, survival was very similar with and without *Artemia* enrichment at high algal density of $10x10^6$ cells ml^{-1}. 'Clear water' culture resulted in poorer larval survival, while on the other hand it showed that *Artemia* enrichment improved survival mainly under 'clear water' or low algal density condition. Meanwhile, increased algal density and *Artemia* enrichment were shown to significantly improve larval development and growth, respectively. The shortest mean development times to PL (20.5 and 21.5 days) were from both 10 $x10^6$ cells ml^{-1} treatments with enriched treatment having the shortest 20.5 days while non-enriched treatment at slightly longer 21.5 days. Meanwhile, all 'greenwater' treatments showed advanced 'Larval Stage Index' (LSI) compared with the 'clear water' treatments. In contrast, dry weights and total lengths of PL from enrichment treatments were heavier or larger. It is concluded that *Artemia* enrichment was most beneficial for larval culture when 'clearwater' method was used or microalgae was added at low concentrations in 'green water' culture.

Newly settled postlarvae (PL) of *M. spinipes* were stocked under three densities of 500 (low), 1000 (intermediate), 2000 PL m^{-3} (high) and with or without shelter, respectively to investigate the effects of stocking density and shelter as well as their potential interactions on the nursery culture of *M. spinipes*. Statistical analysis detected no significant interaction ($P>0.05$) between the two factors of stocking density and shelter within the range tested, the main effects were therefore analysed separately. With the survival results of the two treatments under a same density (i.e. with and without shelter) pooled, it showed that the

low density (500 PL m^{-3}) produced significantly higher mean survival (84.8 %) than the high density treatment (2000 PLm^{-3}) (57.4 %) ($P<0.05$). The intermediate density (1000 PLm^{-3}) generated an intermediate result (74.4 %), which was significantly different ($P<0.05$) from both the high and low density treatments. At the end of culture, the high stocking density also produced significantly heavier juveniles (wet weight: 144.24 ± 0.01 mg ind^{-1}), significantly higher final biomass (13.45 ± 1.39 g) and increase in biomass (8.87 ± 1.28 g) than that of the low density treatment (wet weight: 117.99 ± 0.003 mg ind^{-1}; final biomass: 4.69 ± 0.16 g and increase in biomass: 2.95 ± 0.15 g). Meanwhile, the addition of shelter produced significantly heavier juvenile wet weight (134.3 ± 0.01 vs. 123.61 ± 0.003 mg ind^{-1}) and higher final biomass (11.76 ± 1.84 vs. 8.79 ± 1.12 g). The results suggest that stocking density of 2000 PL m^{-3} improved final production while 500 PL m^{-3} improved survival. On the other hand, under the same stocking densities, shelter addition enhanced juvenile wet weight and final biomass.

In summary, the results of the present study suggest that the Australian native *M. spinipes* is a freshwater *Macrobrachium* species that shares many biological and aquaculture similarities with *M. rosenbergii* but also has some major differences. As it is native to Australia, *M. spinipes* has high potential for aquaculture in the country, therefore should be investigated further for its potential commercial farming in Australia and also possibly overseas.

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Chapter 1. Introduction

1.1 Freshwater Prawn Aquaculture in Australia and Objectives of This Study

Despite huge success of *M. rosenbergii* aquaculture worldwide, due to strict government regulations that prohibit the introduction of foreign species/strains to Australia for aquaculture, the only way to farm freshwater prawns in the country is to develop culture techniques for native species/strains. While the local freshwater prawn species used known as 'eastern form' *M. rosenbergii* was recently renamed as *Macrobrachium spinipes* (Ng & Wowor, 2011) has broad natural distribution in northern tropical regions of Australia (Short, 2004). Unfortunately, several past attempts to commercially culture the species have all failed, reporting various problems, including low hatchery survival, excessive cannibalism, lack of technical expertise and infrastructure to consistently produce postlarvae (Cantrelle, 1988) and diseases (Bergin, 1986; Owens and Evans, 1989). Consequently, ccommercial freshwater prawn farming has in fact never taken place in Australia.

In line with the new findings that wild Australian *Macrobrachium* fauna can be categorized into four genealogically distinguished lineages, i.e. Western Australia (lineage I), Gulf of Carpentaria/Northern Territory (lineage II), Irian Jaya (lineage III) and Papua New Guinea/North east Cape York (lineage IV) (de Bruyn *et al*., 2004b), interest has been regenerated on assessing the aquaculture potential of *Macrobrachium* in Australia,

particularly for native strains that appeared not to have been trialed previously. To this end, wild broodstock of Lineage II strain were sourced from the Flinders River system, North Queensland and a series of studies were carried out to assess its aquaculture potential, which include,

1) Wild broodstock conditioning and inducing out of season spawning (Chapter 2);
2) Improvement of hatchery larval survival and culture techniques (Chapter 3 and 4); and
3) Development of appropriate nursery techniques (Chapter 5), for the Lineage II strain.

As a result, the present thesis includes six chapters. Following an introductory chapter (Chapter 1), each experimental chapter (Chapter 2 to 5) is introduced and discussed individually and the thesis concludes with a final General Conclusion and Discussion chapter (Chapter 6) that overall summarises the results as well as points out future research directions.

1.2 Aquaculture History and Global Production of *Macrobrachium rosenbergii*

Freshwater prawns have been part of the diets of the peoples in Asia and the Far East, particularly in Bangladesh, Pakistan and India, since time immemorial (New, 2010). In these regions, farming of freshwater prawns employed various traditional methods such as "trapping-holding-growing" to introduce young prawns into tanks, "bheris" (or fish

ponds) and rice fields where they are fed and grown to edible size (Ling, 1961). However, availability of wild seeds was the main constraint to such practices of traditional prawn farming and the production level was very low (Ling, 1969). The situation did not change until modern freshwater prawn farming began with the giant freshwater prawn *Macrobrachium rosenbergii* larval culture techniques were successfully developed in late last century. In 1961, Dr. Shao-Wen Ling was the first to report the closure of the life cycle of *M. rosenbergii* after discovering that its larvae required brackishwater for survival (New, 2010). The subsequent work by other researchers, particularly the research group led by Dr. Fujimura in Hawaii, have further developed the hatchery techniques for commercial scale production (Fujimura & Okamoto, 1972). With assistance from FAO, a project aimed at further expanding freshwater prawn farming in a national scale in Thailand produced technical manuals for freshwater prawn culture (New, 2010). Since then, *M. rosenbergii* has been introduced to many countries worldwide for culture and its global aquaculture production has grown rapidly.

Global production of *M. rosenbergii* in 2009 had reached 443,959 tonnes and continued to increase becoming a significant contributor to world aquaculture production (New and Nair, 2012). Based on data from Food and Agriculture Organization (FAO) of the United Nations, production of freshwater prawns referred to as giant river prawn was mostly carried out in Asia with China being the largest producer since 1996 (FAO 2014). Historically Thailand, Taiwan and Vietnam were among the most important producers in the 1970's, 1980's and early 1990's until the addition of Chinese statistics in 1996, which

changed the landscape of the global production of the species (FAO 2014). Chinese production of *M. rosenbergii* was first reported by FAO at 37,000 tonnes in 1996 but increased rapidly and reached 122,933 tonnes with a value of US $585 million in 2011 (FAO 2014) (Table 1).

Table 1: Production (metric tonnes) of Freshwater prawn (*Macrobrachium rosenbergii*) 2002 – 2011 (FAO 2014).

Country	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**
Bangladesh	9,559	10,200	17,123	19,609	20,810	23,240	23,377	26,137	30,636	39,868
Brazil	450	450	363	370	373	230	100	100	100	265
China	98,383	75,376	84,965	85,541	93,695	124,520	127,788	144,467	125,203	122,933
India	30,500	35,870	38,720	42,820	30,115	27,262	12,800	6,600	-	-
Indonesia	400	246	290	1,009	1,199	989	942	696	1,327	617
Malaysia	535	627	317	514	194	246	355	552	619	334
Taiwan	7,026	10,045	10,039	10,515	9,878	8,316	10,058	7,470	6,318	6,460
Thailand	15,393	28,151	32,583	28,740	25,353	32,148	33,189	26,785	25,606	21,080
USA	54	49	38	218	218	200	200	200	200	200
Viet Nam	5,552	5,961	6,247	5,200	5,482	7,900	7,100	7,700	8,190	8,500
Totals	167,852	166,975	190,685	194,536	187,317	225,051	215,909	220,707	198,199	200,257

1.3 Nomenclature and Natural Distribution

The first record of this species was incorrectly described by Herbst in 1792 under the name *Cancer* (*Astacus*) *carcinus,* Linnaeus 1758. The genus name *Palaemon* was then first put forward by Weber in 1795 while in 1879 J.G. De Man discovered a new species in New Guinea which he named *Palaemon rosenbergii*. However *Cancer* (*Astacus*) *carcinus* was the accepted name for subsequent 150 years until Sunier in 1925 pointed out

the incorrect nomenclature, as the first description was based on the American species *C.* (*Astacus*) *jamaicensis.* Sunier proposed a new specific name *dacqueti* for the Indo-west Pacific species of the giant freshwater prawn but did not hold as J.G. De Man's *Palaemon rosenbergii* was the older name than *P. dacqueti* and therefore had precedence. In 1860, the revision by Stimpson placed *Palaemon* species in genus *Macrobrachium* and created a new genus *Leander* for all *Macrobrachium* species, which created much confusion until intervention from the International Commission on Zoological Nomenclature to restore uniformity and decided on the genus name *Macrobrachium* in 1959.

Since then, the taxonomic classification for the species is known as:

Kingdom	Animalia
Subkingdom	Bilateria
Infrakingdom	Protostomia
Superphylum	Ecdysozoa
Phylum	Arthropoda – Artrópode, arthropods, arthropods
Subphylum	Crustacea (Brünnich, 1772) – crustacés, crustáceo, crustaceans
Class	Malacostraca (Latreille, 1802)
Subclass	Eumalacostraca (Grobben, 1892)
Superorder	Eucarida (Calman, 1904) – camarão, caranguejo, ermitáo, lagosta, siri
Order	Decapoda (Latreille, 1802) – crabs, crayfishes, lobsters, prawns, shrimps, crabs, crevettes, écrevisses, homards

Suborder	Pleocyemata (Burkenroad, 1963)
Infraorder	Caridea (Dana, 1852)
Superfamily	Palaemonoidea (Rafinesque, 1815)
Family	Palaemonidae (Rafinesque, 1815)
Subfamily	Palaemoninae (Rafinesque, 1815)
Genus	*Macrobrachium* (Bate, 1868)
Species	*Macrobrachium rosenbergii* (De Man, 1879) – giant river prawn

Direct Children:

Sub-species: *Macrobrachium rosenbergii rosenbergii* (De Man, 1879)

Macrobrachium rosenbergii schenkeli Johnson, 1973

Source: Integrated Taxonomic Information System (ITIS) website (Viewed 25th March 2015)

It is known that the genus *Macrobrachium* includes more than 200 species and the species belonging to the genus are native to all continents except Europe (Holthuis and Ng, 2010). The giant freshwater prawn *M. rosenbergii* is the largest species of all *Macrobrachium,* reportedly grows up to 320 mm in total length for male and 250 mm for female (Brown *et al*., 2010). The natural distribution of *M. rosenbergii* extends from Pakistan across to Southeast Asia, south to Papua New Guinea and northern Australia (de Bruyn *et al*., 2004a), and in some Indian and Pacific Ocean Islands (Mather & de Bruyn, 2003).

Following assessment of the morphological characters of *M. rosenbergii* from different locations, two forms, i.e. 'eastern form' and 'western form', were proposed for the species (De Man, 1879; Johnson, 1973). Further assessment of adult male morphometric and allozyme data suggested these two forms were separated by Wallace's Line, a biogeographic barrier extending north from Indonesia to the east of the Philippines (Fig. 1) (Lindefelser, 1984). More recently, assessment of 16s ribosomal RNA mitochondrial DNA sequences (mtDNA) of wild stocks from 18 wild populations of *M. rosenbergii* revealed significant divergence between specimens, confirming two separate monophyletic clades of *M. rosenbergii*, which supported earlier suggestion of the existence of the two forms (de Bruyn *et al*., 2004a). These authors however proposed Huxley's Line as the biogeographic barrier instead of Wallace's Line (Fig. 1) (de Bruyn *et al*., 2004a) and suggested that these two clades may have been separated as far back as the Miocene Epoch (de Bruyn *et al*., 2004b).

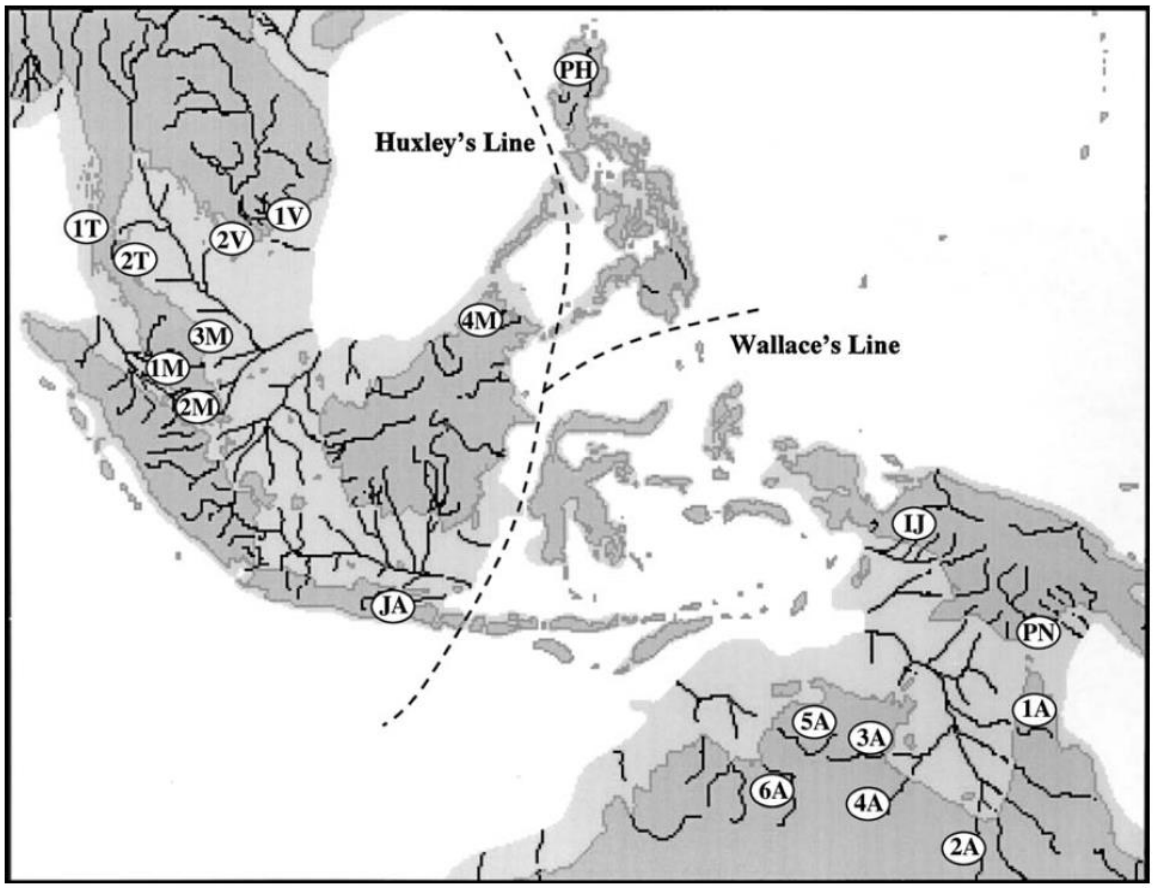

Figure 1: Huxley's Line, a well-known biogeographic barrier demarcating the eastern and western forms of *Macrobrachium rosenbergii*. Also indicating sites where samples were collected for the study. Source: de Bruyn *et al*. (2004a).

The natural distribution of *M. rosenbergii* in Australia ranges from the Fitzroy River basin, Western Australia to the Jeannie River basin, Queensland (Short, 2004) and the first record was from Katherine River, Northern Territory (Short, 2004). Based on the above definition, the Australian *Macrobrachium* fauna belongs to the 'eastern form'. The

origin of the Australian *Macrobrachium* fauna was hypothesized to have a marine ancestor; or alternatively, an estuarine ancestor gave rise to most eastern inland species while a northern origin via Papua New Guinea and Indonesia by island hopping and land bridge crossings (Williams, 1981; Riek, 1959; Bishop, 1967; cited in Murphy & Austin, 2004). More recently, Short (2000) thoroughly investigated the origins of the Australian *Macrobrachium* fauna and hypothesised that the collision of the Sunda Plate and Australian continent lowered sea levels exposed land bridges facilitating movement of *Macrobrachium* to Australia. Other authors however regard the Australian fauna of *Macrobrachium* as a separate 'race' (Hedgecock *et al* 1979; cited in de Bruyn *et al*., 2004b). Following analyses of 16sRNA mitochondrial sequences of *Macrobrachium,* Murphy & Austin (2004) concluded that the endemic Australian *Macrobrachium* fauna did not evolve from a common ancestor but evolved independently of each other and shared close similarities with the Indo-West Pacific *Macrobrachium* fauna.

Short (2000) proposed that the Australian *Macrobrachium* fauna being classified into two races, the northeast and northwest race, with an intermediate form in-between these regions. More recent research by analysing mitochondrial DNA samples collected from Australia, Irian Jaya and Papua New Guinea uncovered phylogenetic relationships among the Australian *M. rosenbergii* fauna and proposed that they be divided into four genealogical lineages: that is, lineage of Western Australia (lineage I), Gulf of Carpentaria/Northern Territory (lineage II), Irian Jaya (lineage III) and Papua New Guinea/North east Cape York (lineage IV) (Fig.2) (de Bruyn *et al*., 2004b).

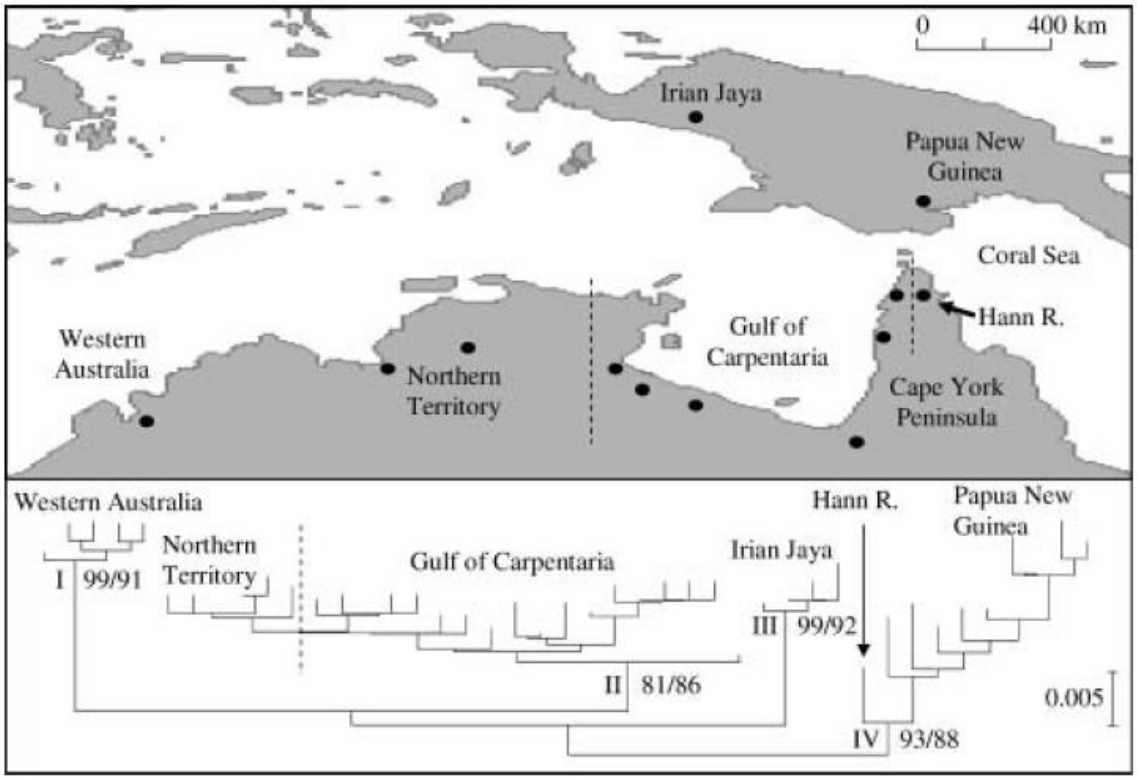

Figure 2: Study area of *Macrobrachium* collected by de Bruyn *et al*. (2004b) indicating sample areas (black dots), lineages (roman numerals) as well as relationships shown on phylogenetic tree and distribution. Source: de Bruyn *et al*. (2004b).

The most recent nomenclature revision of *M. rosenbergii* by Ng & Wowor (2011) suggested a name change to *Macrobrachium spinipes* (Schenkel, 1902) for those previously known as "*Macrobrachium rosenbergii*" found in Sulawesi, Bali, Sumba, Moluccas, Irian Jaya, Papua New Guinea, Australia and the Philippines as they were previously described by Schenkel (1902) as *Palaemon spinipes*. The name *Macrobrachium rosenbergii* remains for those found in other parts of the world. The same

authors proposed that morphological differences between *M. spinipes* in northwestern and northeastern Australia, Papua New Guinea and Philippines may call for further assessment. As a result of this latest assessment, giant river prawn *M. rosenbergii* from Australia will be referred to as *M. spinipes* hereafter in this thesis.

Since global aquaculture of the giant river prawns is based on the 'western form' or *M. rosenbergii* according to recently revised nomenclature by Ng & Wowor (2011), past literature on both biology and aquaculture of the giant river prawns almost exclusively came from *M. rosenbergii*. Hence the information provided in the subsequent sections of this chapter is based on those reported for the 'western form' or *M. rosenbergii* by the new nomenclature. It is assumed that Australian *M. spinipes* closely resembles *M. rosenbergii*.

1.4 General Biology of *Macrobrachium rosenbergii*

1.4.1 Life cycle and habitat

In general, *M. rosenbergii* can be found in lakes, water reservoirs, mining pools, irrigation canals, rivers and its lower reaches influenced by tides and they have been reported to be found 200km inland from the coast in Malaysia (Ling, 1969). Gravid females migrate down rivers to estuaries where they hatch their eggs into free-swimming larvae (Brown *et al*., 2010). Larval development through eleven stages and the final stage larvae metamorphosed and settled as postlarvae (PL) and start migration upstream towards

freshwater (Ling, 1969; Uno & Kwan, 1969; Brown *et al*., 2010). Postlarvae resemble miniature adults and can be active swimmers but are also increasingly benthic and crawl at the bottom (New, 2010). Figure 3 illustrate the life cycle of *M. rosenbergii.*

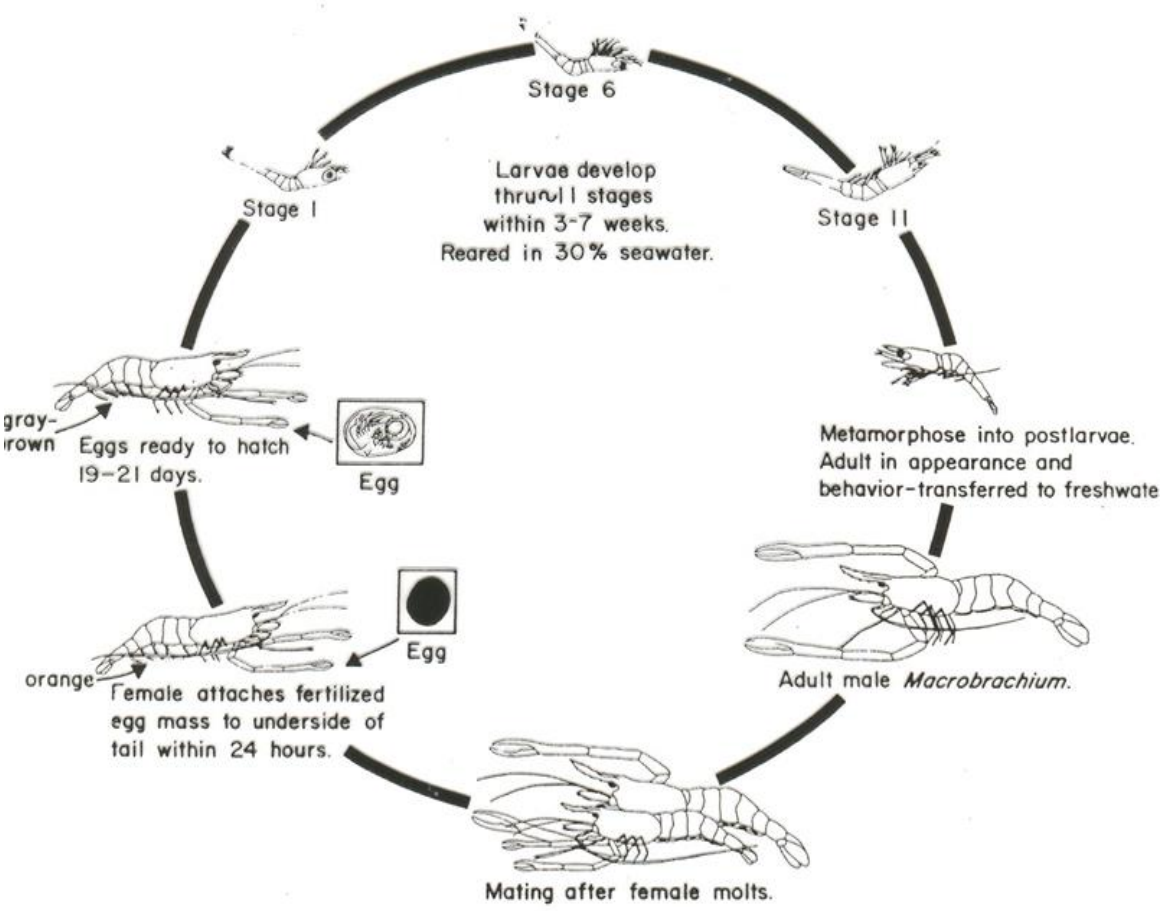

Figure 3: Life cycle of *M. rosenbergii*. (After Sandifer and Smith, 1978).

1.4.2 Diets and appendages for feeding

M. rosenbergii is omnivorous feeding on a wide range of food items, including aquatic worms and insects and their larvae, small molluscs and crustaceans, small fish, nuts and

grains, algae and detritus in its natural environment (Ling & Mercian, 1961; Ling, 1969). Their appendages for feeding include the mandibles, maxillae and maxillipeds. The mandibles are short, heavily calcified molar-like jaws specialised for cutting and crunching; the first and second maxillae are flat, thin and lamelliform. The endopods of maxillae are responsible for handling food towards the mandibles. Maxillipeds located between the mouth and pereiopods and are also involved in food handling. The first two of the pereiopods are chelated where the first pair, smaller than the second, is utilised in capturing food. The second pair of chelated pereiopods is also utilised in food capture, but also used for agonistic and mating behaviour. The chelate finger of the second pereiopod is mobile, covered with a pubescence, characteristic of the species, and ends sharply with an inward tip free of the pubescence (De Man, 1879).

1.4.3 Reproductive biology

1.4.3.1 *Sexual dimorphism and morphotypes of males*

Juvenile males with size larger than 5.9 mm in carapace length can be identified by the appearance of gonopores at the base of the coxae of the fifth pereiopods covered by flaps as well as the occurrence of a lump on the ventral side of the first abdominal somite. Meanwhile, a spinous structure known as the appendix masculina on the endopod of the second pleopod is utilised for copulation. There are three male morphotypes, i.e. blue claw (BC), orange claw (OC) and small males (SM), occurring in sexually mature populations of *M. rosenbergii* (Fig. 4) (Cohen *et al*., 1981; Ra'anan & Sagi, 1985; Kuris *et al*., 1987). Blue claw males are the large, dominant individuals characterised by long

blue, strong spinous second pereiopods with high claw to body length ratio; orange claw (OC) males have a lower claw to body length ratio and medium sized, spineless chelipeds which are sometimes orange; the small males (SM) are often un-pigmented and translucent, possessing the smallest claw to body length ratio. In the intermediate OC males, energy is devoted to somatic growth prior to moult to become BC males while for BC and SM males, energy is mainly channelled for reproduction (Ra'anan & Sagi, 1985).

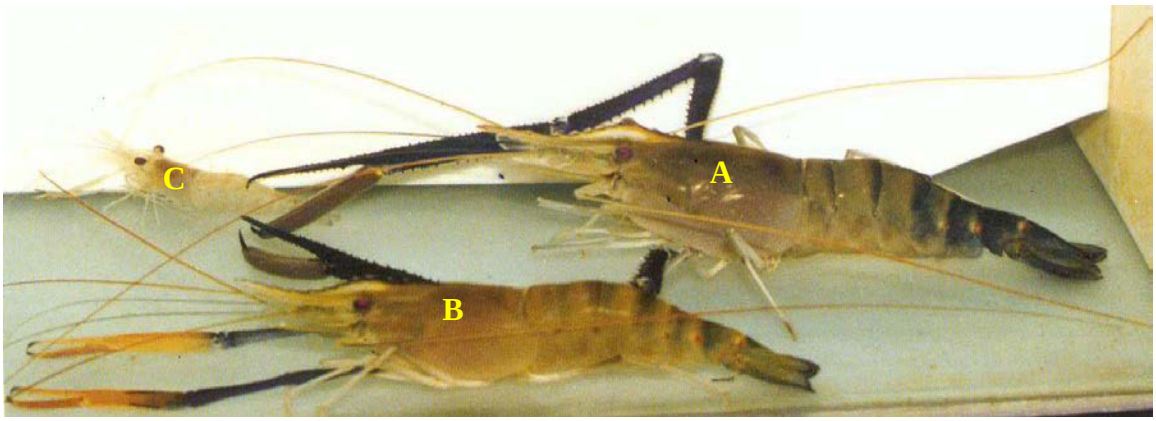

Figure 4: The male morphotypes of *M. rosenbergii*, denoted above are, (A) Blue claw (BC), (B) orange claw (OC) and (C) small male (SM). (Source: Assaf Barki; Plate 3, in New *et al.*, 2010).

In the case of females, gonopores are oval shaped and are located on the coxae of the third pereiopod covered with membranes. Females are generally proportionally smaller in size than males. During breeding season, a brood chamber forms between the first, second and third abdominal pleurae of a sexually mature female with permanent ovipositing setae appearing on the pleopods and the last three pairs of pereiopods. Meanwhile, after a pre-

spawning moult, ovigerous setae occur to secure the eggs to attach to the pleopods for brooding (Brown *et al*., 2010).

1.4.3.2 *Reproduction seasonality*

Berried *M. rosenbergii* reportedly can be found year-round in some tropical regions (Rao, 1991; Daniels *et al*., 2010). However, natural spawning of wild populations of *M. rosenbergii* reportedly peaked during the warmer months (August to October) in Lake Kolleru, India with a temperature between 29-30.5 ºC (Rao, 1991). Peak mating activity in the wild was proposed to occur during the rainy season (New, 1990), which is supported by capture of large number of berried females in Kerala, southwest India with the onset of the rainy season (Varghese *et al*., 1992). However the occurrence of gravid females has also been reported in estuarine waters during cooler months (November to January) in Thailand (Daniels *et al*., 2010).

1.4.3.3 *Mating, spawning, egg hatching and larval development*

Based on external observation of size and colour of developing ovary which can be seen through the carapace, ovarian developmental stages of *M. rosenbergii* larvae has been described (Damrongphol *et al*., 1991; Chang & Shih, 1995). Stage I is an early development stage represented by a small white mass inside the carapace while maturity at stage V is characterised by reddish ovary extending from the rear of the eyes to the anterior of the first abdominal segment. Upon reaching stage V, the females are ready to mate. The spermatozoa of *M. rosenbergii* are non-motile and enclosed in a packet called

the spermatophore, rich in glycoproteins and acid mucopolysaccharides to keep it viable till fertilisation occurs.

The mating behaviour of *M. rosenbergii* was first described by Ling (1961). Mating commences only after a female undergoes a pre-spawning moult which usually occurs overnight. Courting behaviour includes the stroking movements of the antennae, the male touching the female chelipeds; raising the body until the female accepts the male. The latter then holds the female and commences brushing or cleaning the female ventral thoracic region with its pleopods. As a result, the female becomes torpid and copulation follows with the male depositing a sperm mass in the ventral side of the thoracic area and subsequently he dismounts the female (Ling, 1969).

Females extrude eggs soon after copulation through gonopores, guided by the ovipositing setae to the abdominal brood chamber. Fertilisation is external and occurs during the movement of the eggs to the brood chamber. Ovigerous setae on the pleopods secure the eggs in the abdominal region during embryonic development, which last about 3 weeks at temperatures 26 - 28 ºC (Ling, 1969). The newly laid eggs are usually bright orange, as they develop, the eggs change colour to light gray (12^{th} day) and then to dark brown when fully developed around the 16^{th} to 17^{th} day after spawning (Ling, 1969).

Eggs hatch into free-swimming larvae swimming ventral side up and undergo eleven developmental stages (I – XI) (Ling, 1969; Uno &Kwon, 1969) before moulting into

postlarvae that resemble miniature adults (Tab 2 and Fig. 3). The eleven larval stages have been described by Ling (1961; 1969) and Uno & Kwon (1969) from laboratory reared larvae (Tab.2) and are also illustrated in Fig. 3 and 5.

Table 2: Key characters of larval developmental stages of *Macrobrachium rosenbergii* fed *Artemia* nauplii. Modified from Brown *et al*., (2010), Ling (1961) and Uno & Kwon (1969).

Larval Stage	Recognized characters
1	Sessile eyes
2	Supra-orbital spine, eyes stalked laterally and rudimental articulation of uropod
3	Doral rostrum tooth, sixth abdominal somite separated, uropod appear
4	Two dorsal rostrum teeth, uropod biramous with setae, chromatophores appear on second pereiopod merus
5	Posterior margin of telson narrower and elongated, chromatophores prominent on mid-ventral abdomen
6	Buds appear on pleopods, telson more narrower and elongated terminally
7	Pleopods biramous and bare; outer antenullar flagellum with four aesthetes on folded appendix
8	Pleopods biramous with setae; incomplete chelae
9	Endopods of pleopods with appendices internae
10	Three or four dorsal rostrum teeth; middle dorso-lateral spines of telson disappear; first and second pereiopods fully chelated
11	Rostrum teeth on half of upper dorsal margin
Postlarva	Behaviours of swimming and locomotion as adult

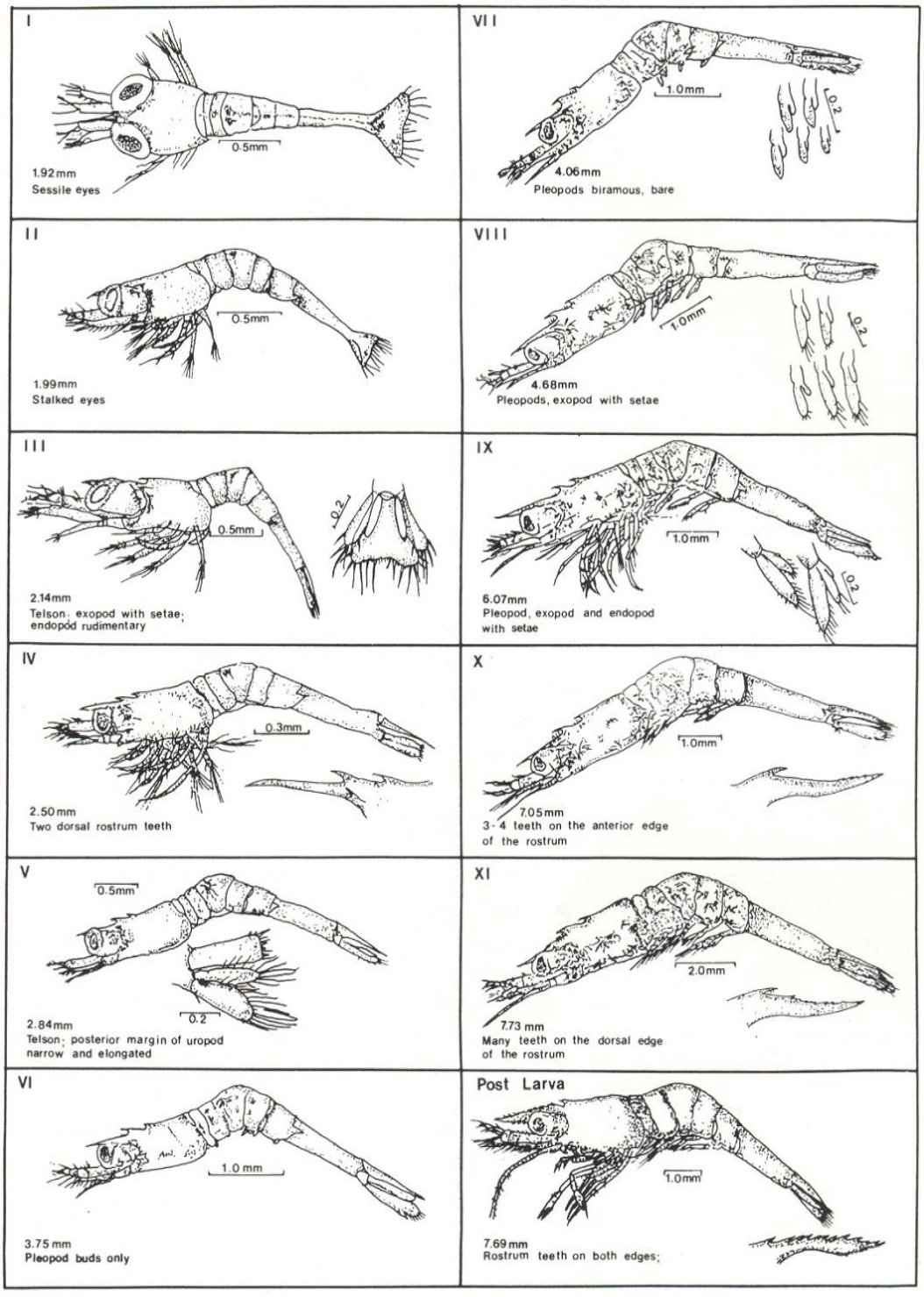

Figure 5: Larval development of *M. rosenbergii*. (Source: Malecha, 1983).

1.5 *M. rosenbergii* Aquaculture Practices

1.5.1 Broodstock management

In countries where *M. rosenbergii* aquaculture is widely practiced, berried females are usually collected directly from culture ponds for hatching larvae for hatchery rearing; often females with brown or greyish-brown eggs are selected as the coloration indicate late embryonic development (Malecha, 1983; Daniels *et al*., 2010). However, in some hatcheries, selection of broodstock females that are either close to spawn by setal examination of pleopods (Peebles, 1977) or close to pre-mating moult by external examination of ovarian development through the carapace, have also been practiced (Chang & Shih, 1995; Sagi & Ra'anan, 1985).

Fecundity of *M. rosenbergii* generally increases with female size (Patra, 1976; Malecha, 1983; Ang and Law, 1991; Cavalli *et al*., 2000; Habashy, 2013). In commercial hatcheries, large females are normally selected and depending on various reports, female size of 75 to 100g (Mohanta, 2000), 45g (Daniels *et al*., 2010) or ≥ 30g (Sandifer & Smith, 1978) have been used. However, Doyle *et al*. (1983) suggested that selection of smaller, fast growing females at 3 months of growout exerts a positive effect on the growth of the progeny than females at 6 months. Yet, the use of smaller females has been resisted by farmers as they produce smaller number of eggs and therefore more berried females are required for spawning (New, 1995).

Blue claw (BC) males have traditionally been selected for mating as reproductively mature females are attracted to BC males and the latter utilise less energy to manipulate the female for insemination (Daniels *et al*., 2010). Small males (SM) are reproductively mature and active, and can perform a 'sneak' copulation with a newly moulted female guarded by a BC male (Ra'anan & Sagi, 1985). Although it has been reported BC males exclusively contain mature sperm (Sagi *et al*., 1988; Sagi & Ra'anan, 1988), their selection for copulation may have a reproductive disadvantage as testicular index reduces with age and less viable sperm might be available for fertilization (Sureshkumar & Kurup, 1998; Daniels *et al*., 2010). Daniels *et al*., (2010) suggested stocking of 'pre-transforming OC males' (TSOC) (Sagi & Ra'anan, 1988) with BC males for mating with broodstock females as sexual activity of the OC males spans 3-4 months before their next moult to BC when they could grow larger than the largest BC male, a phenomenon known as the "leap-frog" growth pattern (Ra'anan & Cohen, 1985).

Generally, *M. rosenbergii* are tolerant of temperatures between 18 ºC to 34 ºC but optimal is believed to be between 27 ºC and 32 ºC (Daniels *et al*., 2010). At lower temperatures, egg development takes longer, providing conditions for fungal growth and eggs may drop off (Daniels *et al*., 1992) while temperature above 30 ºC encouraged protozoan and other micro-organism growth (New, 2002). Under experimental conditions, increasing temperature from 28 ºC to 32 ºC enhanced growth rate and frequency of reproductive moulting (Chavez Justo *et al*., 1991). Daniels *et al*. (2010) reported that photoperiod had no significant role in reproduction, however, a 12L: 12D light regime enhanced the

frequency of reproductive moulting under laboratory conditions (Chavez Justo *et al*., 1991). Salinity is believed to improve hatchability of eggs, and maintenance of berried females in brackishwater of 3 to 5 ‰ at 25 – 30 ºC, reportedly stimulated egg development (New, 2002). In practice, brackish water with a salinity range from 5 to 12 ‰ can be utilised for maintenance of berried females (Daniels *et al*., 2010).

As a warm water species, plummeting water temperatures in temperate climate necessitates collection of broodstock from production ponds during autumn harvesting and over-wintered in temperature-controlled facilities (Sandifer and Smith, 1978; Daniels *et al*., 2010). Over-wintering of *M. rosenbergii* broodstock often requires a well-managed holding system until the next production cycle. To supply 500,000 larvae for the next production cycle, it is estimated such a system needs to be stocked with at least 440 females (45 g) and at least 110 males (Daniels *et al*., 2010). Such estimation is based on assumption that survival will be at 50 % and with 5 % of females spawning at any one time (Daniels *et al*., 2010). The holding system is maintained at a temperature > 25 ºC with optimal water quality to maximise the health of the broodstock. Structures or shelters should be provided as they can greatly reduce cannibalism and increase survival. Varghese *et al*. (1992) reported that the best sex ratio for oviposition and larval production were between 3.75 - 4.75 females per male; similarly the recommendation for commercial hatcheries was 1 male: 4 females when blue claw (BC) males were utilised (Sureshkumar & Kurup, 1998). Malecha (1983) also suggested a male to female broodstock sex ratio of 1:4 or 1:5 but did not specify the male morphotype.

1.5.2 Larval culture

1.5.2.1 *Larval feeds*

Newly hatched *M. rosenbergii* larvae depend on yolk for survival, however, larvae start feeding on zooplankton at stage II. In hatcheries, they are fed newly hatched *Artemia* nauplii (Dhont *et al*., 2010). *Artemia* has been utilised as the first feed for the larvae because the larval gut of *M. rosenbergii*, especially the anterior midgut and the hepatopancreas, are not well developed until stage V to VI (Deru, 1990), hence early larvae require the digestion of live prey that provide exogenous enzymes (Jones *et al*., 1993). Early larval stages of *M. rosenbergii* are thought to capture food mainly by chance encounter (Moller, 1978), therefore constant movement of *Artemia* nauplii in the water column also increases chance of encounters and they are thought to provide a good feeding stimulus for larvae (Daniels *et al*., 1992). While early larvae are carnivorous, they become more omnivorous from stages VII onwards when formulated feed can be used as supplemental feeds (Barros & Valenti, 1997). The high cost and uncertainty in supply of *Artemia* cysts have led to development of inert supplemental feeds such as egg custard and microbound diets for *M. rosenbergii* larvae (Dhont *et al*., 2010). It was suggested that inert feed particles of 0.3 mm be introduced for larval culture at day 10 to 12 old larvae (Corbin *et al*., 1983; New 2002); or feeds of 0.2 to 0.4 mm particle size being given from stage VII onwards (Barros &Valenti, 2003). Microbound diets are still under development although progress has been made with several investigators claiming larval consumption on such diets as early as stage IV (Daniels *et al*., 1992) or complete replacement of

Artemia from stage V to PL (Kovalenko *et al*., 2002). However, commercial hatcheries still rely on *Artemia* in combination with supplemental feeds (Dhont *et al*., 2010).

1.5.2.2 *'Greenwater' vs 'Clearwater' larval culture method*

Although various methods have been developed and practiced in different countries, the basic requirements for hatchery culture of *M. rosenbergii* were summarised in an FAO manual authored by New (2002). Following the pioneer work by Ling who carried out research that closed the life cycle of *M. rosenbergii* for the first time in Malaysia (Ling, 1969), the subsequent introduction of *M. rosenbergii* to Hawaii facilitated the development of mass larval culture method during the 1960's (Fujimura & Okamoto, 1972) known as the 'greenwater' method. The 'greenwater' method encourages the growth of phytoplankton in the culture medium to promote larval survival and to improve water quality (New, 2010). However, since then, the alternative 'clearwater' method where phytoplankton is omitted from larval culture has gradually taken over, especially in commercial hatcheries (Hudon *et al*., 1989; New, 2010). 'Clearwater' systems have been favoured recently most likely due to their efficiency and ease of management as compared to 'greenwater' method, as the latter not only requires skilled personnel and facilities for algal culture, but also because if it is not managed properly, high pH and excessive algal blooms or crashes can cause high larval mortality (Hudon *et al*., 1989).

Hatcheries operating on the 'clearwater method' for larval culture use either flow-through or closed recirculating systems. The former requires regular water exchange to remove toxic wastes in the culture water produced by larval and *Artemia* metabolism and decomposition of faeces and dead organisms (Valenti *et al*., 2010). These hatcheries are more commonly located near coastal areas to allow good access to supply of seawater. Some inland hatcheries also utilise artificial seawater however this is not recommended by New (2002) in his culture manual, as the optimum ionic composition for freshwater prawn is not known. High water exchange rates in flow through systems require the use of large volumes of water and may cause shock to larvae, which are among the main setbacks to this method (Valenti *et al*., 2010). Closed recirculation systems were designed to minimise water loss while water quality was maintained by mechanical filters that continuously removed particles in the system while biological filters convert toxic ammonia to less toxic nitrate. Such systems also allowed for better disease management (New, 2002; Valenti *et al*., 2010). Recirculation systems are especially popular for inland hatcheries where either artificial seawater is used or seawater is transported long distances. Recirculation systems are believed to be better for larval hatcheries as they save on water and cost, less environmental imprint and are believed to be more productive (New, 2002; Valenti *et al*., 2010).

1.5.2.3 *Stocking density, duration of larval culture and other culture conditions*

Larval stocking densities vary greatly among hatcheries, ranging from 50 – 100 larvae/ L in backyard hatcheries to 50 – 1000 larvae/ L in large modern commercial hatcheries (New, 1990). Larval density may be adjusted later by dilution or transfer to another tank if the initial stocking density was high and early larval survival was good (New, 2002; Valenti *et al*., 2010). For example, AQUACOP (1983) reported initial stocking densities of between 60 - 70 larvae/ L, however, they stocked larvae with initially low water levels and subsequently gradually increased the water level to decrease the larval density or to reduce larval density during later culture phases by transferring some larvae to another larval tank for culture (New, 2002; Dhont *et al*., 2010).

Among other important culture conditions, the temperature adopted for larval culture was more consistent mostly between 28 – 31 °C (New, 2002). Duration of larval culture under such temperature is typically between 30 to 35 days (Uno & Kwon, 1969; AQUACOP, 1983; Mallasen & Valenti, 1998; New, 2002; Valenti & Tidwell, 2006). It has been reported that at between 24 to 26 °C larvae take a substantially longer time to reach metamorphosis while temperature over 33 °C caused high mortalities (New 2002; Valenti *et al*., 2010).

Co-existence of larvae of several developmental stages commonly occurs after a while of larval culture. The appearance of the first postlarvae reportedly ranged from 18 to 35 days (Ling 1969; Uno & Kwon, 1969; Manzi & Maddox, 1977; Malecha *et al*., 1980). Apart from temperature, the larval culture duration is also related to culture method used, feeds

and feeding regimes as well as other culture conditions (New, 1990; Valenti *et al*., 2010). While salinity for hatching is preferably 0 – 5 ‰ (Ling, 1969), once eggs hatched into larvae, the salinity should be increased to about 12 ‰ by either adding prepared saline water or transferring larvae to separate rearing tanks filled with 12 ‰ brackish water (New, 2002). Optimal larval rearing pH was suggested by Ling (1969) and New (2002) to be 7.0 to 8.0, which was confirmed by Mallasen and Valenti (2005) who found larvae tolerated high levels of total ammonia (NH_4-N) at pH 7 and 8 but survival decreased at higher pH of 9.0.

Light requirement during larval culture is not well studied although larvae are known to be positively phototatic but avoid direct sunlight (Ling, 1969). Most 'clearwater' systems minimise the use of light to prevent phytoplankton blooms and New (2002) recommended covering 90 % surface of larval flow-through tanks kept outdoor. However, larval production in dark green walled tanks placed near a window was reportedly higher than tanks placed in semi-darkness condition, suggesting a light requirement for larval culture (AQUACOP, 1977). New (2002) recommended the use of natural indirect light although artificial sources could be alternatives. Light intensities reported for successful larval rearing are between 250 and 6500 lux for commercial hatcheries, although the requirement of spectral quality of light is not well understood (New, 1990). In temperate zones, light is considered important for larval culture in late winter when light intensity is reduced and the use of artificial light to supplement natural light is suggested to provide sufficient light intensity for larval feeding (Tidwell and D'Abramo, 2010). Photoperiod

effects on larval culture are not clearly understood (Valenti *et al*., 2010) although under a continuous light regime (L:D 24:0), larval development and food consumption of *M. rosenbergii* reportedly increased (Lin, 1997). The colour of the larval culture tank was suggested by Yasharian *et al*. (2005) to affect larval survival as well, with red and green coloured tanks producing significant higher larval survival than white and blue tanks.

The un-ionised form of ammonia (NH_3) is toxic to larval prawns at a concentration of 8 mg/ L at pH 9, causing high mortality and this also slowed development although larvae of *M. rosenbergii* could tolerate high levels of total ammonia (NH_4-N) (Mallasen & Valenti, 2005). The equilibrium relationship between NH_3 - NH_4^+ was also affected by temperature, salinity but pH was most influential, therefore pH was a critical water parameter for larval prawn that should be monitored during culture (Mallasen & Valenti, 2005). Larvae of *M. rosenbergii* are also sensitive to nitrite (NO_2^-) with increasing ambient nitrite (NO_2-N) to 16 mg/ L, delayed larval development, retarded larval growth and caused larval mortality (Mallasen & Valenti, 2006). In contrast, nitrate (NO_3-N) did not proffer toxicity problems to larvae up to 180 mg/ L and concentrations as high as 1000 mg/ L (NO_3-N) were tolerated although Larval Stage Index (LSI) and weight gain decreased at high nitrate levels (Mallasen *et al*., 2003).

1.5.3 Nursery

Nursery stage of culturing postlarvae (PL) to juveniles in freshwater is sometimes practiced, particularly for locations where climate was a constraint (Ling, 1969). In

general, the nursery phase for *M. rosenbergii* was between 45-60 days in temperate climates and operated on recirculating systems (D'Abramo *et al*., 1995; Tidwell *et al*., 2005). This is considered critical for culture of *M. rosenbergii* in temperate regions by getting a "head start" for growout (Tidwell & D'Abramo, 2010). In tropical areas, the nursery phase can hold postlarvae from one week up to 3 months (New, 2002).

Nurseries are either indoor or outdoor depending on the climate, location and management strategy (New, 2002; Coyle *et al*., 2010). Indoor nurseries also known as primary nurseries in tropical areas are used for adapting postlarvae to freshwater and maintain postlarvae before stocking into growout ponds (New, 2002; Coyle *et al*., 2010). The indoor nursery in temperate areas commences in late winter operating on water recirculation systems (Valenti and Tidwell, 2006). Outdoor nurseries, also known as secondary nurseries, operate year round, are stocked with postlarvae from hatcheries or larger juveniles from primary nurseries and reared for 4 to 10 weeks until they reach sizes of 0.8 – 2.0 g (New, 2002).

The stocking density is dependent on the duration of the nursery phase and size of juveniles required for stocking growout ponds (Zimmermann and Sampaio, 1998). The stocking density in temperate regions vary broadly and have not been well defined, reportedly from < 200 to > 6000 PL/ m^2 of bottom area (Tidwell *et al*., 2005). Stocking density for tropical nurseries range from 350 to 5000 PL/ m^2 for one week holding (Alston, 1989), or 1,500 – 2,000 PL/ m^2 for up to one month holding (New, 2002).

Stocking densities recommended in New (2002) were 2,000 PL/ m^3 in tanks with substrates and 1,000 PL/ m^3 without substrate, assuming the nursing period is 20 days. However a reduction in density should apply if the postlarvae are to be kept beyond 20 days.

Addition of artificial substrates into nursery tanks is recommended to increase area available to postlarvae subsequently increasing survival and production (Valenti & Tidwell, 2006). The types of substrates used include plastic mesh (Smith & Sandifer, 1975), horizontal layers of plastic sheeting (Coyle *et al*., 2003) and plastic netting (Sandifer *et al*., 1983). The design of the substrate is owed to the 'edge effect' of the behavioural characteristic of *M. rosenbergii* juveniles as they prefer the edges of the substrate; the horizontal and vertical planes of substrates offer more surface edges available to prawns (Sandifer *et al*., 1983).

Nursery also allows size grading that minimizes size variation during harvesting (Tidwell & D'Abramo, 2010). Size variability can be better managed through frequent sampling and grading in smaller nursery ponds than stocking postlarvae directly into large production ponds (New & Singholka, 1985). In sub-tropical and tropical regions, the use of nurseries maximises the efficient use of growout ponds for larger prawns (New, 2002), as well as efficient use of labour, water and land, and makes three production cycles yearly possible (Coyle *et al*., 2010).

Feeding postlarvae in nurseries generally use either commercially available pellet feeds or freshly prepared feeds (New, 2002; Coyle *et al*., 2010). Caution is advised when using fresh feeds as water quality can deteriorate quickly with the breakdown of fresh food, requiring more water exchange and this imposes increased costs (New, 2002; Coyle *et al*., 2010). Feeding frequency is either once or twice a day with feed ration adjusted according to prawn biomass (New, 2002). The optimal protein requirement for juvenile *M. rosenbergii* was reportedly 30 to 35 % (Balazs & Ross, 1976; Millikin *et al*., 1980) while dietary lipid requirements can be as low as 2 % (Sheen & D'Abramo, 1991).

Optimal water temperature for nursery rearing was reported between 27 to 33 °C for *M. rosenbergii* (Sandifer *et al*., 1983; New, 2002). Higher temperatures were shown to increase growth rates, particularly weight gain (Niu *et al*., 2003). Water pH was critical especially in outdoor nurseries where algal blooms can substantially influence pH (Coyle *et al*., 2010). A preferred pH range of 6.5 to 9.0 was suggested for outdoor nurseries and any increase in pH above 9.0 as a result of photosynthetic activity of algae required water exchange to lower pH (Sandifer *et al*., 1983). For indoor recirculating nurseries, New (2002) recommended similar water quality management as freshwater hatcheries i.e. pH 7.0 to 8.0, dissolved oxygen (DO) > 5 ppm. A photoperiod experiment has shown higher survival of nursery raised juvenile prawns under a constant light regimen (L24:D0) (72 %) than L12:D12 (59 %) and constant darkness (L0:D24) (58 %) conditions (Tidwell *et al*., 2001).

1.5.4 Growout

The most commonly practiced growout culture system for *M. rosenbergii* is semi-intensive growout, in which stocking density generally ranges between 4 to 20 individuals/ m² with productivity of 500 to 5000 kg/ ha/ yr (Valenti & New, 2010). Semi intensive culture in ponds can either be continuous culture or batch culture or a combination of the two, which is also known as the 'combined system' (New 2002). A continuous culture system involves regular stocking of postlarvae and harvesting of marketable size prawns. However a setback to this practice is that inefficient cull harvesting often allows some large dominant prawns to remain in the system and impact negatively on the newly stocked postlarvae (New, 2002). For batch culture, ponds are stocked only once to grow prawns to medium size and then ponds are completely harvested (New, 2002). In a combination of batch and continuous system, large prawns are selectively harvested and by the end of culture cycle, the ponds are drained followed by a complete harvest (New, 2002). Optimal stocking densities vary with culture system and increasing stocking density generally reduces the average weight at harvest (Siddiqui *et al*., 1997). The addition of substrates has also been shown to increase productivity for monoculture in temperate farms (Cohen *et al*., 1983; Ra'anan *et al*., 1984).

Grow-out feeds currently are farm made due to the extensive and semi intensive nature of farming including commercial feeds of other animals (D'Abramo & New, 2010). Feeds generally constitute 40 – 60 % of operational costs for the growout of *M. rosenbergii* (D'Abramo & Sheen, 1994). Reduction of costs involves replacement of animal protein,

usually fishmeal, with cheaper plant protein (Tidwell *et al*., 1993) or fertilizing the culture ponds to encourage growth of macroinvertebrates as feeds for prawns (Tidwell *et al*., 1995). Feed rates are normally based on percentage of body weight of cultured prawns (D'Abramo & Sheen, 1994). For example, it has been suggested that when PL's are stocked for batch culture, feeding ration should be 20 – 10 % of body weight initially but this can be gradually reduced to 2 % close to harvest time (New, 2002).

Chapter 2. Trial to induce out-of-season spawning of *Macrobrachium spinipes* (Schenkel, 1902) by temperature and photoperiod manipulation

2.1 Abstract

Inducing spawning of *Macrobrachium spinipes* outside its natural breeding season (November to March) was investigated by temperature and photoperiod manipulation, the effects of such manipulation on survival and moulting of adult female prawns were also evaluated. Fifteen mature female prawns were subjected to each of four experimental conditions: water temperature kept at 24, 27 and 30 °C, respectively with photoperiod adjusted to 14.5 h light: 9.5 h dark and a control with ambient temperature + ambient photoperiod. The experiment ran from late May to mid-August over 84 days. No mortality was recorded throughout the experimental duration for the 24, 27 °C treatments and the control (survival 100 %) while a single mortality was recorded on day 66 for the 30 °C treatment (survival 93.3 %). All moults were identified as common moults with no reproductive moult recorded in any of the treatments. The intermoult period between common moults decreased with increase in temperature and was the shortest in the 30 °C treatment (30.1 ± 0.50 days) and the highest for the control (44.4 ± 10.62 days). Intermoult period in the 30 °C was significantly shorter ($P>0.05$) than all other treatments except for the 27 °C treatment (34.3 ± 11.03 days). Despite a lack of reproduction moult by the temperature and photoperiod manipulation, results of the study showed high tolerance of *M. spinipes* broodstock to winter holding condition at low temperatures,

which in turn also suggests good potential for grow-out culture of the species in cooler waters.

2.2 Introduction

Based on broodstock of Malaysian origin, the freshwater prawn *Macrobrachium rosenbergii* is now cultured worldwide with world production exceeding 221,000 tonnes in 2007 (New, 2010). However, two forms of *M. rosenbergii* have been recently identified, divided into an 'eastern form' and a 'western form' by a well known biogeographic barrier, Huxley's line (De Bruyn *et al*., 2004a). Furthermore, the latest nomenclature assessment of *M. rosenbergii* has suggested a name change to *Macrobrachium spinipes* for the strain previously known as "*Macrobrachium rosenbergii*" from Sulawesi, Bali, Sumba, Moluccas, Irian Jaya, Papua New Guinea, north Australia and the Philippines, while the name *Macrobrachium rosenbergii* remains for populations in South East Asia and those cultured worldwide (Ng & Wowor, 2011). Hence, the widely studied and cultured form of *M. rosenbergii* mainly of Malaysian origin conforms to the 'western form' while the Australian/ Papua New Guinea fauna of *M. spinipes* conforms to the 'eastern form' (Ng & Wowor, 2011) with four identified lineages (de Bruyn *et al*., 2004b). The diversity of wild *Macrobrachium* stocks as a result of these new developments has suggested future breeding programme potential and conservation for *Macrobrachium* (Mather & de Bruyn, 2003). Biological information currently available has been almost exclusively based on the widely commercially farmed 'western form' or *M. rosenbergii* (hereafter *M. rosenbergii* will be used exclusively for

the ‘western form’) based on the most recent nomenclature revision (Ng &Wowor, 2011) but little is known about the Australian ‘eastern form’ or *M. spinipes*.

Based on past publications, the natural spawning of *M. rosenbergii* occurs in the tropics throughout the year but peaks during the summer season (Rao, 1991) at the onset of the rainy season (New, 1990). For *M. rosenbergii* introduced to cold climate conditions, spawning only occurred in summer but spawning can be effectively induced by either maintaining water temperature between 27 to 32 °C (Daniels *et al*., 2010) or photoperiod manipulated for 12L: 12D (Chavez Justo *et al*., 1991). A combination of the two factors is utilised in the artificial spawning of *M. rosenbergii* and photoperiod is set up according to the local conditions (Adiyodi, 1985; Chavez Justo *et al*., 1991). Both unilateral and bilateral eyestalk ablation have also been demonstrated to induce spawning of *M. rosenbergii,* although it is important that ablation should be carried out during a certain stage of moult cycle to achieve the best results (Okumura & Aida, 2001; Buitrago & Salazar, 1993).

Prior to this study, *M. spinipes* natural breeding season in tropical Queensland was revealed to be between November to March, in a pilot study was further carried out to investigate inducing out-of-season spawning during September to November at JCU (Lober, 2006). Water temperature was controlled constantly at 30 °C and photoperiod kept at 14L: 10D for a 70 day trial, which resulted in 100 % reproductive moulting and spawning between October and November 2006 with an average intermoult period

between reproductive moults of 25.33 ± 1.53 days (Lober, 2006). At the JCU research facility, *M. spinipes* has shown to cease spawning after April, coincident with the end of the rainy season and leading into the cooler season in tropical North Queensland. This is in stark contrast to the western Malaysian strain which reportedly is capable of year round breeding in the tropics (New, 1990; Rao, 1991), providing supporting biological evidence for the recent nomenclature revision of these into two different species of *M. rosenbergii* and Lineage II *M. spinipes*, from north Queensland, respectively (Ng &Wowor, 2011).

Reproduction in freshwater prawns (*Macrobrachium*) commences with ovarian development in the carapace, which is followed by ecdysis (or reproductive moult) and occurrence of ovigerous setae on the pleopods, spawning of eggs occurs soon after and eggs extruded was secured into the abdominal brood chamber for brooding. In contrast, a non-reproductive or common moult is absent of these physical indicators and is associated with somatic growth only (Ling, 1969; Nagamine & Knight, 1980).

In efforts to investigate the aquaculture potential of the Australian native *M. spinipes*, successful hatchery culture was achieved by the JCU crustacean research group during the natural breeding season between November to March. Clearly, if out-of-season spawning of *M. spinipes* can be similarly induced by temperature and photoperiod manipulation as has been reported for *M. rosenbergii,* it could enhance the aquaculture potential in Australia for *M. spinipes*. Based on an earlier pilot experiment that successfully induced out-of-season spawning approximately one month before the onset of natural spawning

season of the Australian *M. spinipes* (Lober, 2006), the current experiment was conducted to investigate the possibility of inducing out-of-season spawning immediately after the end of the natural breeding season by temperature and photoperiod manipulation under laboratory conditions. Additionally, the effects of temperature on moulting and growth of adult females, in particular their tolerance to winter low ambient temperatures, was also carried examined.

2.3 Methods and Materials

2.3.1 Source of female prawns

Experimental prawns were collected in April from a wild population near Burketown in North Queensland, Australia. The prawns were transported to the Marine and Aquaculture Research Facility Unit (MARFU), James Cook University in North Queensland, Australia and individuals held in several 3000 L oval tanks until experimentation commenced in May. Samples of wild prawns were also sent to Queensland University of Technology (QUT) and confirmed as belonging to Lineage II from rivers flowing into the Gulf of Carpentaria (de Bruyn *et al*., 2004b). This group have recently been given the revised name of, *Macrobrachium spinipes* (Ng &Wowor, 2011).

2.3.2 Experimental design and general procedure

A total of 48 adult female prawns (24.74 ± 8.13 g) were acclimated to 27 °C and a photoperiod set at 14.5 h L: 9.5 h D for 14 days prior to experimentation. Data on average carapace length, (CL - from the tip of the rostrum to the posterior edge of the carapace,

Plate 1), orbital length (OL - from the base of the left eye socket to the posterior margin of the carapace, Plate 2), total length (TL - from the tip of the rostrum to the posterior edge of the telson, Plate, 3) and wet weight (g) of the prawns were recorded prior to the experiment and data are shown in Table 1. OL and CL data were obtained using a digital calliper (Mitutoyo Digimatic, Model No. CD-6" PS) and a tape measure was used to measure total length. Weight was measured using an electronic scale (Ohaus Scout Pro SP401).

Three treatments with water temperature controlled at 24, 27 and 30 °C were set up with photoperiod manipulated at 14.5 h L: 9.5 h D for all treatments (Plate 5) while ambient temperature + ambient photoperiod treatment was used as the control (Plate 6). All treatments were in triplicate with each replicate including 5 female prawns caged in baskets and placed inside a 500 L round tank filled with approximately 380 L water (Plate 4). All experimental prawns were marked numerically with a white permanent marker on their carapace and were kept individually in marked baskets covered by rigid mesh (l x w x h = 440 x 281 x 254 mm) (Fig. 4). Each prawn was remarked after moulting. Replicates for the 3 control temperature treatments (± 1 °C) were placed randomly inside an air-conditioned room (Fig. 5) while the control was kept outdoors under shade (solar weave) (Fig. 6). Water temperatures in indoor tanks was maintained at constant levels using a submersible aquarium heaters (300 W) and daily temperature fluctuations were monitored using a minimum – maximum thermometer. Timers were used to control photoperiod at 14.5L: 9.5D, simulating the day-length during summer in tropical north Queensland.

Illumination was provided by the main light sources of four fluorescent lights (58 W) and additional four table lamps (40 W). The latter was timed to switch on between 05:30 - 07:00 and 18:30 - 20:00 to simulate dawn and dusk when the fluorescent lights went on and off. Light intensity measured over the water surface of experimental tanks during the light phase ranged between 603 to 1291 lux.

Aeration was provided to all the tanks and 50 % water exchange was carried out every 3 days while a 70 % water exchange was conducted every 15 days. New water used for water exchange was pre-heated to the assigned temperatures in separate tanks and pumped across to corresponding experimental tanks with submersible pumps. Feed was administered *ad libitum* daily alternating between fresh feed (squid, mussel and shrimp) and grower pellet for the black tiger prawn (*Penaeus monodon*) (protein 30 %). Uneaten feed and faeces was siphoned out daily. The study lasted for 84 days from late May to mid-August during the non-breeding season of the Australian *M. spinipes*.

Daily data collection included temperature; common or reproductive moult and mortality. Measurements for CL, OL, TL (mm) and wet weight (g) were taken five days after moulting occurred to allow the new exoskeleton to calcify.

2.3.3 Data Analysis

The different inter-moult periods among treatments were analysed using one-way ANOVA and a Tukey's honest test was used for multiple comparisons of significance.

Differences were considered significant at P<5% level. Data analyses were carried out using statistics package, SPS Version 16.0.

2.4 Results

2.4.1 Water quality parameters

Water quality parameters were generally maintained within tolerable ranges across the experiment duration (Table 3). For the control in which prawns were kept under ambient conditions, lower temperatures were generally recorded early in the morning and the lowest temperature recorded was 12 °C while the highest was 26 °C with an average temperature of 21.4 ± 2.5 °C for the whole experimental duration.

2.4.2 Survival

Survival of the female prawns was very high and similar for all treatments, no mortalities were recorded from the 24, 27 °C treatments and the control (100 % survival) while a single mortality was recorded in the 30 °C treatment during the late stage of the experiment on day 66.

2.4.3 Moulting and intermoult duration

There were no reproductive moults recorded throughout the experimental duration for all treatments. However it was observed that some prawns in the three control temperature treatments exhibited light orange colouration on the dorsal side of the carapace behind the rostrum after moulting but this was always short-lived. Since both ovarian maturation and spawning were not observed in any treatments across the whole experiment duration, all moults were defined as common moults.

The number of common moults was highest in the 30 °C treatment (4.6 ± 1.2) followed by 24 °C (4.0 ± 0.8) and 27 °C (3.5 ± 0.7) while the control had the least occurrence of moults (2.8 ± 0.9) (Table 4). As a result, the average intermoult duration between common moults was the shortest for the 30 °C treatment (30.1 ± 0.5 days), which was followed by 27 °C (34.3 ± 11.0 days) and 24 °C treatment (36.1 ± 11.3 days) while the intermoult period for the control was significantly longer than in all experimental treatments (50.8 ± 2.6 days) ($p>0.05$) (Table 4). Of the 3 temperature control treatments, the intermoult period was not significantly different ($P<0.05$) between 30 and 27 °C treatments but was significantly different between the 30 and 24 °C ($P>0.05$) treatments.

Figure 6 shows fluctuations in ambient water temperature over the experimental duration and the times common moult occurred. Ambient water temperature fluctuated greatly over the duration of the experiment but was generally lower during July and August, the cool months in North Queensland (Fig. 6). By the end of the study, only six of the fifteen prawns in the control had undergone a second moult however it was interesting to note that moulting did occur even with temperatures plunging to a minimum of around 12 °C and an average of about 15 °C during late July (Fig.6).

2.4.4 Growth

The final carapace length of prawns in the 30 °C treatment was the largest, showing the highest growth (61.79 ± 1.95 mm) amongst all treatments, while the lowest growth was recorded in the control (55.66 ± 1.66 mm) (Table 5), which was significantly different

($P>0.05$) from all other three treatments. Similar results were obtained for total length with the control showing significantly shorter total length (125.67 ± 2.34 mm) compared with other treatments. Finally, the highest orbital length was recorded in the 24 °C (34.96 ± 2.56 mm) and the least in the control (32.94 ± 1.71 mm), however no significant differences were detected among all treatments ($P<0.05$) (Table 5). It should be noted that the orbital length is considered as the most reliable length data as it was not affected by damage to the rostrum or tail rot disease during experimentation.

Weight gained was recorded in all treatments except for the control (Table 5). The highest weight gained was recorded in the 24 °C treatment (3.86 ± 3.39 g) while the control recorded an average weight loss of -1.53 ± 5.66 g. It should be noted that some individuals in the control did show positive growth but the average was negative, which led to a large standard deviation. As a result, there was no significant difference ($P<0.05$) detected among all treatments (Table 5).

Figure Legend

Figure 6: Temperature and common moults recorded for *M. spinipes* adult females under ambient condition.

Figure 6

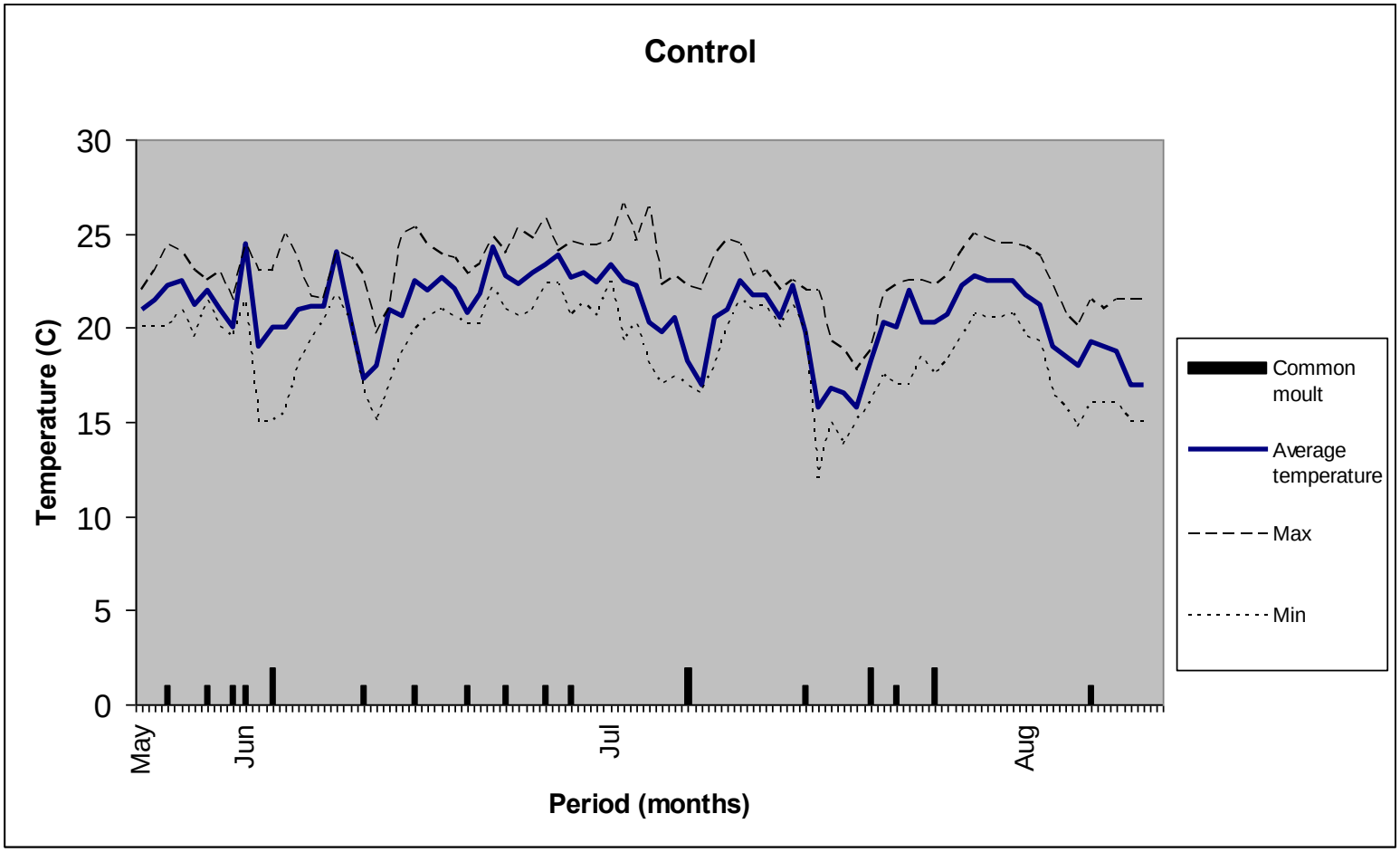
Control
30
25
20
15
10
5
0
Temperature (C)
May
Jun
Jul
Aug
Period (months)
Common moult
Average temperature
Max
Min

Table 3: Water quality parameters during the experimental duration.

Treatments	30 °C	27 °C	24 °C	Control
Dissolved oxygen (mg/ L)	4 – 4.7	4.1 – 5.8	4.6 – 6.7	4.8 – 7.7
pH	6.5 – 7.9	6.5 – 7.9	6.4 – 7.9	5.9 – 9.1
Ammonia (mg/ L)	0 – 0.5	0 – 0.5	0 – 0.5	0 – 1.5
Nitrite (mg/ L)	0 – 0.8	0 – 2.0	0 – 2.0	0 – 0.5
Nitrate (mg/ L)	0 – 60.0	0 – 60.0	0 – 20.0	0 – 8.0
Temperature (Mean± SD)(°C)	30.1 ± 0.4	27.0 ± 0.6	23.9 ± 0.5	21.4 ± 2.5

Table 4: Numbers of common moults and intermoult duration of female *M. spinipes* kept under different temperatures during 84 days experimental duration. Different superscript letters indicate significant differences between treatments (P<0.05).

Treatment	Number of common moult	Intermoult period for common moult (days)
30 °C	4.6 ± 1.2^{a}	30.1 ± 0.5^{a}
27 °C	3.5 ± 0.7^{a}	34.3 ± 11.0^{ab}
24 °C	4.0 ± 0.8^{a}	36.1 ± 11.3^{b}
Control	2.8 ± 0.9^{a}	44.4 ± 10.6^{c}

Table 5: Final carapace, orbital, total lengths and weight gained (Mean ± SD) (mm) of *M. spinipes* kept under different temperatures during 84 days experimental duration. Different superscript letters indicate significant differences between treatments (P<0.05).

Treatment	Carapace length (mm)	Orbital length (mm)	Total length (mm)	Weight gain (g)
30 °C	61.79 ± 1.95 [a]	34.27 ± 1.85 [a]	131.6 ± 2.95 [a]	3.19 ± 1.60[a]
27 °C	58.0 ± 3.27 [a]	33.44 ± 0.76 [a]	130.0 ± 4.04 [a]	3.05 ± 1.74 [a]
24 °C	61.18 ± 1.41 [a]	34.96 ± 2.56 [a]	135.4 ± 4.04 [a]	3.86 ± 3.39 [a]
Control	55.66 ± 1.66 [b]	32.94 ± 1.71 [a]	125.67 ±2.34 [b]	-1.53 ± 5.66 [a]

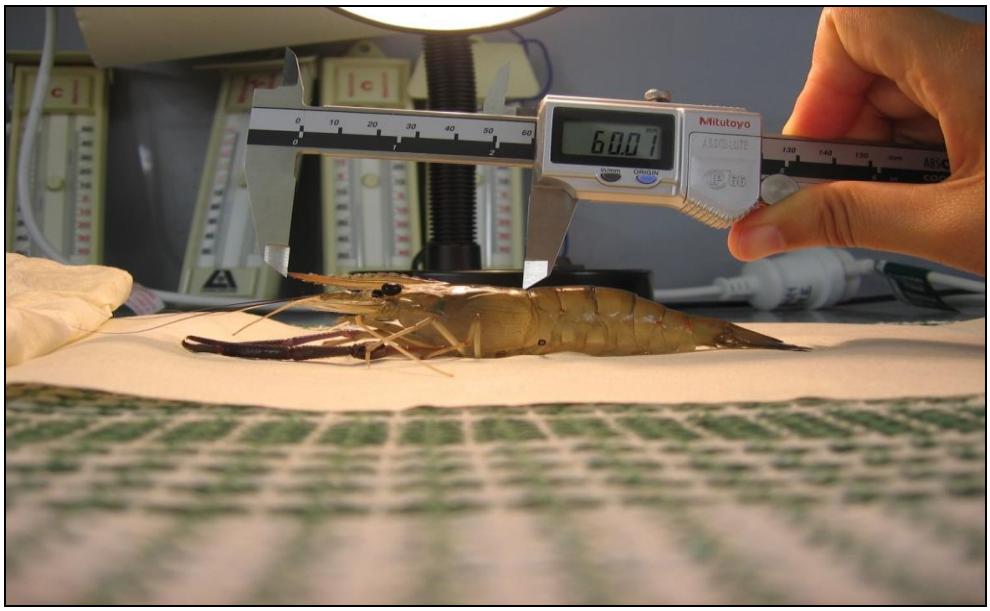

Plate 1: Measuring carapace length (CL).

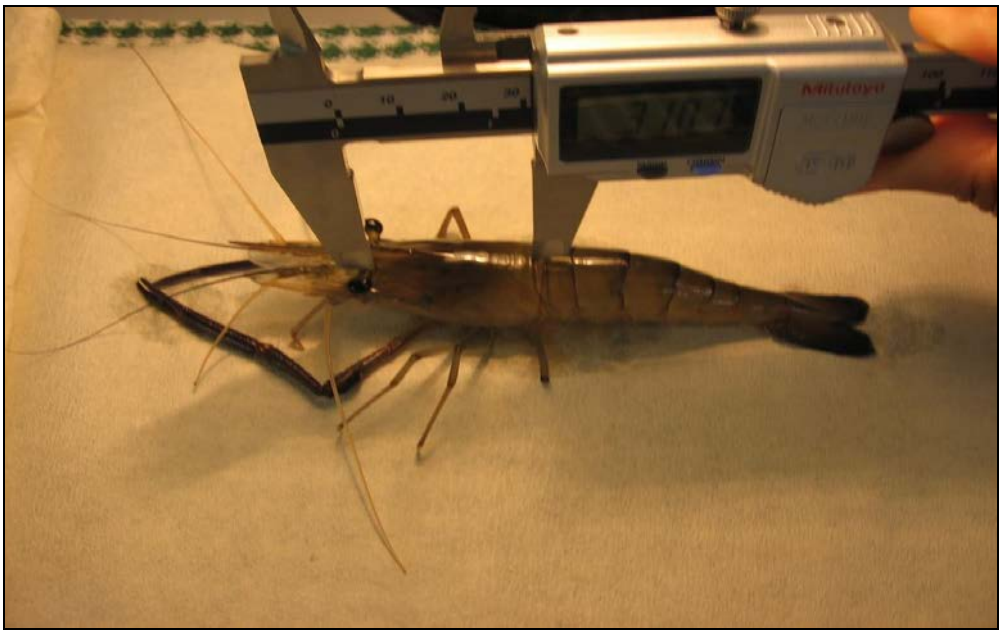

Plate 2: Measuring orbital length (OL).

Plate 3: Measuring total length (TL).

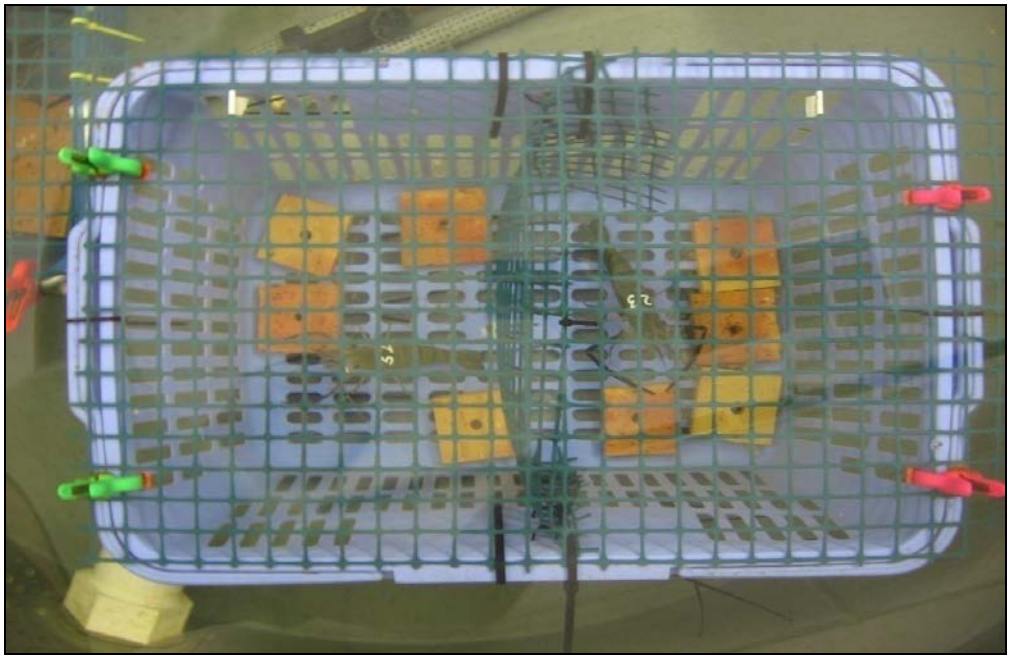

Plate 4: Marked prawns in a mesh basket.

Plate 5: Controlled temperature treatments kept in a laboratory.

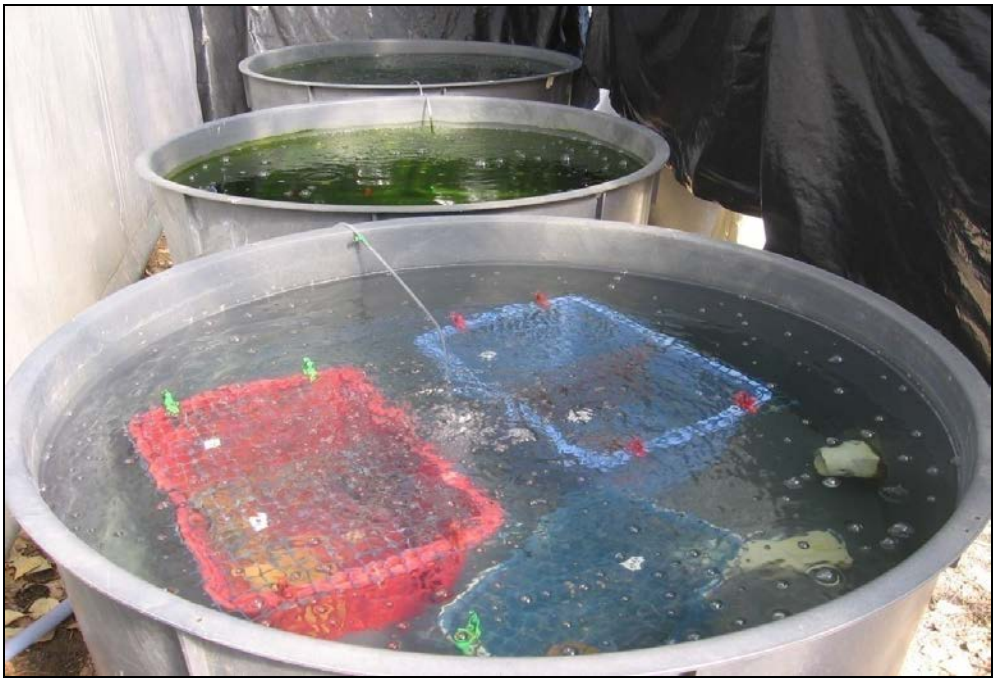

Plate 6: The control kept outdoor under ambient condition.

2.5 Discussion

Inducing out-of-season spawning of Australian *M. spinipes* by temperature and photoperiod manipulation immediately after its natural breeding season (November to March) during May to August was not successful in this study. However, in a pilot trial of out-of-season spawning induction for the species was conducted in September to November leading to its natural breeding season was more successful with spawning being induced approximately one month prior to the natural breeding season (Lober, 2006). The difference most likely can be explained by the different timing of the induction being carried out as for the present study, the induction was conducted after the natural spawning season and through the coolest months in north Queensland when induction of spawning is probably much more difficult.

It has been reported that longer day length (15L: 9D) enhanced reproduction of *M. rosenbergii* (Chavez Justo *et al*., 1991). Similarly, the spawning rate of female freshwater crayfish, *Cherax quadricarinatus*, reportedly increased significantly from 15 % when light periods were < 12 hours to 35 % when light phases were longer than 12 hours with a peak spawning under 14 hours of light (Rouse & Yeh, 1995). Meanwhile maintaining water temperature between 27 to 32 °C has also been reported to successfully induce out-of-season spawning in *M. rosenbergii* (Daniels *et al*., 2010). In practice, manipulation of temperature and photoperiod is usually combined to enhance the effects (Adiyodi, 1985). The longer day length (14.5L: 9.5D) applied in current study however in combination with 3 higher than ambient temperatures failed to induce out-of-season spawning of

Australian *M. spinipes* soon after the end of the natural breeding season. Such a result is clearly different from those reported for *M. rosenbergii* (Chavez Justo *et al*., 1991; Daniels *et al*., 2010).

Despite unsuccessful spawning induction of *M. spinipes* in this study, survival of female prawns in small cages was very high. In fact among all treatments, only a single mortality was recorded under ambient condition throughout the prolonged 84 days experimental duration despite temperatures dipping as low as 12 °C for the control. This is a very positive outcome for potential broodstock to be held over winter under commercial scale culture. Despite time consuming maintenance, the method of caging proved very effective in maintaining broodstock survival as cannibalism was a major problem in communal culture of broodstocks in tanks, leading to loss of broodstock particularly during the moult stage. Caged broodstock were also generally healthier with intact appendages. However further study is needed to evaluate the cost effectiveness of the caging, particularly regarding logistics, applicability in large scale situation and to assess the additional costs involved.

In addition to high survival, it was also interesting to observe that while moulting interval increased significantly at low temperatures, moulting did not cease but continued to occur even when temperatures plunged to as low as 12 °C. Similar tolerance to low temperature has also been reported for *M. nipponense,* which has been suggested to survive as low as 4

°C but growth ceased below 15 °C (Maclean & Brown, 1991). Good cold water tolerance of *M. spinipes* suggests potential of the species to be cultured under cooler conditions.

As the current study showed that unlike *M. rosenbergii*, spawning induction by temperature and photoperiod manipulation for the Australian native *M. spinipes* was unsuccessful during the months soon after the end of the natural breeding season, this presents a challenge for future research. Further investigation adopting alternative techniques for spawning induction, including trialing unilateral or bilateral eyestalk ablation (Okumura & Aida, 2001; Buitrago & Salazar, 1993), should be conducted. Clearly, future study to acquire more biological information about *M. spinipes* will be necessary to evaluate its aquaculture potential as well as to establish optimal culture conditions for the species.

Chapter 3. Effect of microalgae concentration on larval survival, development and growth of an Australian giant freshwater prawn *Macrobrachium spinipes (Schenkel, 1902)*.

3.1 Abstract

The giant freshwater prawn *Macrobrachium spinipes* is distributed naturally across tropical Australia. As a result of failed past attempts to farm local *M. spnipes* and strict regulations preventing introducing species from overseas to Australia for aquaculture, currently no freshwater prawn farms exist in the country. Recent studies showed that Australian *M. spinipes* is genetically distinguished from their widely cultured overseas counterparts and multiple strains exist within Australia. This has generated renewed interest in culture of native *M. spinipes* in Australia. The present study investigated the effects of microalgae *Nannochloropsis* sp. addition and concentration on larval survival, development and growth of an Australian freshwater prawn *M. spinipes* (lineage II). Newly hatched larvae were reared to postlarval (PL) stage under the condition of no algae addition ('clear water') and four *Nannochloropsis* concentrations of 2.5, 6.25, 12.5 and 25 x 10^5 cells/ ml. All treatments were in quadruplicate and each replicate had 30 larvae stocked in a 5 L vessel. Larvae were fed 3 *Artemia*/ ml throughout with 100 % water exchange daily. The results showed that larval survival to PL at the two higher algae concentrations of 12.5 and 25 x 10^5 cells/ ml (70.8 % and 63.3 % respectively) were significantly higher ($P<0.05$) than those of lower algae concentrations of 2.5 and 6.25 x 10^5 cells/ ml and the 'clear water' treatments (26.7 %, 35.0 % and 30.0 % respectively).

Meanwhile, the fastest mean development to PL (30.6 days) registered at the highest algal density was 14 days shorter than that of the 'clear water' treatment (44.3 days). Larval development at the two higher algal densities were significantly shorter than that of the 'clear water' treatment and larval development of the highest algal density was further significantly faster than those of the two lower algal densities (40.1 and 40.0 days) ($P<0.05$). The mean dry weights of newly settled PL of the two high algal density treatments were also significantly heavier ($P<0.05$) than those of the lowest algal density and the 'clear water' treatments. The results have shown that the addition of *Nannochloropsis* at appropriate levels substantially improved performance of larval culture of the Australian Lineage II *M. spinipes*, it meanwhile suggested that the Australian native strain has a promising future for aquaculture.

3.2 Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, has long been part of the diet of the peoples of Asia and the Pacific who greatly value its flavour. The extensive farming of *M. rosenbergii* employing various traditional methods has a long history in Southeast Asia where it occurs naturally (Ling, 1969). The development of modern culture techniques for the species in the 1960's and 70's led to the introduction of the species to many countries and farming of the species is now wide spread throughout the world wherever appropriate climate and freshwater impoundments exist (New, 2002; 2005).

The natural distribution of *M. rosenbergii* extends from Pakistan across to Southeast Asia, south to Papua New Guinea (de Bruyn *et al.*, 2004a) and in some Indian and Pacific Ocean islands (Mather & de Bruyn, 2003). In Australia, the giant freshwater prawn is widely distributed across tropical northern regions and is endemic to the west of the Great Dividing Range (Short, 2004). Several attempts in the past to grow local *M. spinipes* commercially in the country failed, and reported various problems, including low hatchery survival, excessive cannibalism, lack of technical expertise and infrastructure to consistently produce postlarvae (Cantrelle, 1988) as well as diseases that affected commercial operations (Bergin 1986; Owens and Evans, 1989). As a consequence of such failed attempts and strict government regulations that prohibit introduction of foreign species/ strains to Australia for aquaculture, commercial freshwater prawn farming is currently non-existent in the country.

The worldwide upsurge in *Macrobrachium* culture in the past decade has prompted research interest again in Australia, particularly on the problematical hatchery phase. These new efforts were further encouraged by recent reports, which through the analyses of 16sRNA, divided natural populations of *M. rosenbergii* into 'eastern' and 'western' forms, demarcated by Huxley's Line, a biogeographic barrier running between Borneo and Sumatra extending north into the Philippines (de Bruyn *et al.*, 2004a). Based on this finding, *M. spinipes* in Australia constitutes the 'eastern' form, differing from the widely cultured 'western' form of mainly Malaysian origin (de Bruyn *et al.*, 2004a; Ng and Wowor, 2011). Further analysis of mitochondrial DNA revealed that within Australia, wild stocks of *M. spinipes* can be categorized into four genealogically distinguishable lineages, i.e. Western Australia (lineage I), Gulf of Carpentaria/Northern Territory (lineage II), Irian Jaya (lineage III) and Papua New Guinea/North east Cape York (lineage IV) (de Bruyn *et al.*, 2004b).

In line with these new findings, in a new attempt to assess the aquaculture potential of Australian native strains of *M. spinipes*, wild broodstock from Lineage II were sourced from the Flinders River system, North Queensland and larval culture trials were carried out based on techniques developed for the 'western form' (New, 2002). As both 'clear water' and 'green water' methods have been used for larval culture of the 'western form' *M. rosenbergii* and no clear verdict has been made as to which was superior (New, 2002), both methods were trialled on several larval culture runs for the Australian Lineage II *M. spinipes* in identical tanks to compare their relative merits. Of interest, contrary to the

current trend of most commercial hatcheries overseas that opt for the 'clear water' method (New, 2002), results of some preliminary trials suggested that the 'green water' method consistently produced significantly better results for the Australian Lineage II *M. spinipes*. Anecdotal observations further suggested that larval performance was linked to the density of microalgae added. Hence, the present study was designed to assess quantitatively the effects of algae addition on larval survival, development and growth of the Australian Lineage II *M. spinipes*.

3.3 Materials and Methods

3.3.1 Source of broodstock and larvae

Broodstock prawns were collected from the Flinders River system and its tributaries, near the Gulf of Carpentaria (latitude 17°52.522; longitude 140°46.837), North Queensland, Australia. Prawns were transported overnight in 200 L black plastic drums with aeration to the Marine and Aquaculture Research Facility Unit (MARFU), James Cook University (JCU), Townsville, Queensland. Upon arrival at MARFU, broodstock prawns were held in recirculating 2,500 L tanks at a female to male ration of 4-5 : 1. Prawns were fed daily to excess on shrimp, mussel, squid and a formulated feed (36 % crude protein; 6 % crude fat and 3 % fibre) designed for black tiger prawn *Penaeus monodon* (Ridley Aqua-feed, Australia). Samples of wild prawns were sent to Queensland University of Technology (QUT), Brisbane for identification and were confirmed as belonging to Lineage II characteristic of rivers flowing into the Gulf of Carpentaria (de Bruyn *et al.*, 2004b).

Development of ovaries and spawning by female prawns were monitored closely. Berried females were transferred to a salinity of 5-8 psu (brackish water) for incubation and egg development monitored to predict the date of hatching. On the day of hatching, broodstock females were removed from the hatching tank after larvae had hatched and salinity in the tank had been increased to 12 psu. Newly hatched larvae were held for 1 h at salinity 12 before being collected and transferred to experimental containers for the experiment.

3.3.2 Experimental design and setup

Five larval culture treatments were setup with concentrations of green microalgae *Nannochloropsis* sp. set at five levels of 0, 2.5, 6.25, 12.5 and 25 x 10^5 cells/ ml. All treatments were in quadruplicate where each replicate consisted of a 5 L round clear plastic container (diameter = 215 mm; depth=177 mm) stocked with 30 randomly selected newly hatched larvae in salinity 12 ± 1 psu. Replicates of various treatments were organised in a random block design inside water baths set at 30.0 ± 1.5 °C. Each container was covered with a clear plastic lid to prevent larvae jumping out during the late larval stages and gentle aeration was provided to each container via a fine-tipped glass pipette inserted through the lid. Photoperiod was set at 14.5L: 9.5D throughout the experiment and larvae were cultured from the day of hatching (day 0) until they either reached postlarval stage (PL) or death, in all replicates.

Throughout the experiment, larvae were fed a ration of 3 *Artemia*/ ml with 100 % water exchange carried out daily. Water was exchanged in the morning where mortality was recorded and surviving larvae from each replicate were then transferred to an identical container with freshly prepared food (3 *Artemia*/ ml and designated concentration of *Nannochloropsis*) and 12 psu water. Water salinity was pre-adjusted by diluting natural seawater of salinity 33-36 with dechlorinated tap water using a refractometer (Shibuya Salinometer S-10). Water quality parameters, including ammonia, nitrite, nitrate, pH and DO, were measured weekly. Over the period of the experiment, the ammonia, nitrite and nitrate ranged from 0-0.3, 0-0.1 and 0-10 mg/ L respectively while pH fluctuated between 7.4 to 7.9 and DO between 5.7 to 6.3 mg/ L.

Microalgae *Nannochloropsis* sp. was mass cultured in 3000 L tanks at JCU's algal culture facility using a commercially available fertiliser (AQUASOL, Yates Ltd, New South Wales, Australia). *Nannochloropsis* cultures were generally re-inoculated every 7-10 days. During the experiment, a stock solution was made up daily by selecting a *Nannochloropsis* sp. culture at its exponential phase of growth and the water diluted to salinity 12 by mixing it with dechlorinated freshwater in a 20 L container with strong aeration. Samples were then taken from the stock solution and the algal density counted using a hemocytometer under a high power microscope. *Nannochloropsis* concentration of the stock solution was estimated by averaging the concentrations of five 1 ml samples. The volume of stock solution required to make up a desired concentration of microalgae for each treatment was calculated using the equation:

$$C_1V_1 = C_2V_2$$

where C_1 was the designated algal concentration for a particular treatment and V_1 was the total volume required for daily water exchange of the treatment; C_2 was the algal density of the stock solution and V_2 was the volume of the stock solution required to make up the designated algal concentration for the particular treatment. The required volume of the stock solution was subsequently measured and diluted with pre-adjusted 12 psu brackish water to make up the total volumes required for daily water exchange. Meanwhile, *Artemia* cysts (INVE Inc., Thailand) were hatched daily in salinity 18 and newly hatched nauplii were harvested early morning on the following day and their density counted before being fed to the larvae directly without enrichment.

Every 3 days, sixteen larvae (4 from each replicate) were sampled randomly from each treatment and their developmental stages identified under a microscope according to Uno and Kwon (1969). The larvae were placed in a small pool of water during the stage identification and returned to the original culture promptly after staging. This process has been shown previously to not lead to larval mortality when operated properly. Once postlarvae were found in a replicate during the daily check, they were removed from the cultures and euthanized by quick freezing. Larvae were then measured for carapace length (mm) using a microscope equipped with a camera (Leica). They were subsequently dried individually in a 60 °C oven for 24 h before being weighed for dry weight using a Cahn C-33 microbalance (0.001 mg).

3.3.3. Data Analysis

Based on results of larval staging, larval stage index (LSI) was calculated according to Manzi *et al*. (1977) and Mallasen and Valenti (2006):

$$LSI = (\Sigma S_i \times n_i)/N$$

Where, S_i = larval stage (i =1-11; representing each larval stage); n_i = number of larvae in stage S_i; N = total number of larvae examined. The survival, mean development time from hatching to PL, mean carapace length of PL were analysed using one-way ANOVA while mean dry weights of newly settled PL were log transformed prior to analysis with one-way ANOVA. Tukey's test was employed to detect significant differences between treatments at the 0.05 significance level (Sokal and Rohlf, 1995). All data analysis was carried out using statistic's package, SPSS Version 16.0.

3.4. Results

3.4.1 Larval survival

Figure 1 shows percentage survival of newly hatched larvae to PL stage in different treatments. The effects of *Nannochloropsis* addition on larval survival were evident: the highest survival (70.8 %) was obtained with the highest microalgae concentration of 25 x 10^5 cells/ ml, followed by the second highest microalgae concentration treatment of 12.5 x 10^5 cells/ ml (63.3 %). Significantly lower ($P<0.05$))survival was recorded in both the 'clear water' treatment (30.0 %) and the two lower microalgae density treatments of 2.5 and 6.25 x 10^5 cells/ ml (26.7 % and 35.0 %), respectively. Statistical analyses showed that larval survival in the two high algal treatments were significantly better ($p<0.05$) than

that of the other 3 treatments with either low or no algal addition, however, no significant differences ($p>0.05$) was detected between the two highest algal treatments and among the 3 low algal treatments (Fig. 7).

Of interest, a plot of mean daily larval survival showed that for the first 5-6 days, larval survival in all treatments was very high and not substantially different among treatments. Differences in survival was evident however, over the period of day 7 to 20, during which larval survival in the two high algal treatments remained > 80 % while those in the low algal and 'clear water' treatments declined substantially (Fig. 8).

3.4.2 Larval development

Larval development, as measured by mean time required to develop to PL stage, showed a clear positive relationship with higher algal concentration (Tab. 6). The fastest development registered in the highest algal density (25×10^5 cells/ ml) was approximately 14 days shorter than that in the 'clear water' treatment. Statistical analysis confirmed that larval development in the highest algal concentration was significantly faster than in either of the two lower algal concentrations (2.5 and 6.25×10^5 cells/ ml) and the 'clear water' treatment while larval development in the second highest algal treatment (12.5×10^5 cells/ml) was also significantly shorter than that in the 'clear water' treatment ($P<0.05$) (Tab. 6). Furthermore, larval development appeared to be more synchronised at the highest algal concentration of 25×10^5 cells/ ml, as indicated by a substantially lower standard deviation (30 ± 2.6 days) (Tab. 6).

The larval stage index (LSI) calculated based on larval samples taken across the culture period showed a general trend of higher LSI value with increased algal concentration. Significant differences in LSI occurred as early as day 5, when the LSI in the highest algal concentration was significantly higher than those in the lowest algal concentrations and the 'clear water' treatment (Tab.7). The first PL appeared on day 20 and 22 for higher algal treatments of 12.5 and 25 x 10^5 cells/ ml respectivelywhile the first PL was found in the lower algae concentration of 6.25 x 10^5 cells/ ml and the 'clear water' treatment, on day 28 and 35 respectively (Fig. 8). At 2.5 x 10^5 cells/ ml, the first and second PL's were observed relatively early on day 22 and 23 respectively, however, unlike in other treatments where additional PL's were found on following days, the 3rd PL in this treatment only appeared 7 days later, on day 30. The LSI data showed that the first metamorphosis to PL generally occurred at LSI $\leq$ 9 in all treatments except for the highest algal density treatment where it was 10. This supports the contention that development was more synchronised in the highest algal concentrations.

3.4.3 Postlarval dry weight and carapace length

Dry weight of newly settled PL's generally increased with elevated microalgal concentration (Tab. 6). Dry weights of PL's in the two high algal concentrations were the same at 0.852 mg, and this was 20.6 % heavier than the lowest dry weight (0.676 mg) recorded for the lowest algal density and in the 'clear water' treatments. The dry weight of PL's from the 6.25 x 10^5 cells/ ml treatment were intermediate at 0.704 mg. Statistical

analysis showed that PL dry weights in the two high algal concentration treatments were significantly higher ($p<0.05$) than those from the lowest algal densities and the 'clear water' treatment. In contrast, no significant differences in mean carapace lengths of PL were found ($P>0.05$) among other treatments (Tab. 6).

Figure Legends

Figure 7: Cumulative larval survival from hatching to postlarvae of an Australian Lineage II freshwater prawn *Macrobrachium spinipes*, cultured under different concentrations of *Nannochloropsis* sp Data are presented as mean ± SD (n=4). Different superscripted letters indicate significant differences ($P<0.05$).

Figure 8: Daily percentage survival of larvae of an Australian Lineage II freshwater prawn *Macrobrachium spinipes*, cultured under different concentrations of *Nannochloropsis* sp. Note that postlarvae survival were not included and the survival line of a treatment terminated on the day all larvae in the treatment became PL or dead.

Figure 7

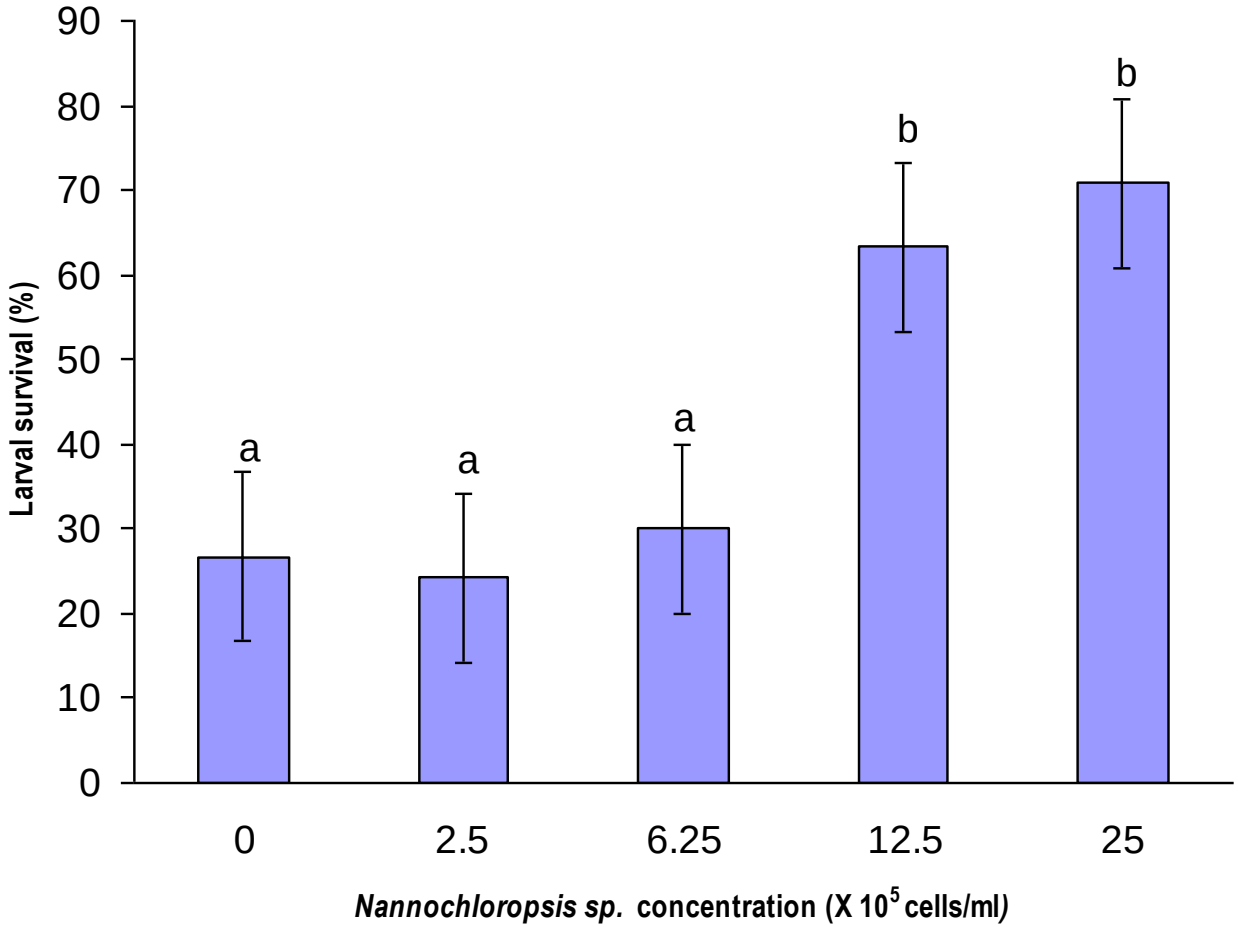
90
80
70
60
50
40
30
20
10
0
Larval survival (%)
a
a
a
b
b
0
2.5
6.25
12.5
25
Nannochloropsis sp. concentration (X 10^5 cells/ml)

Figure 8

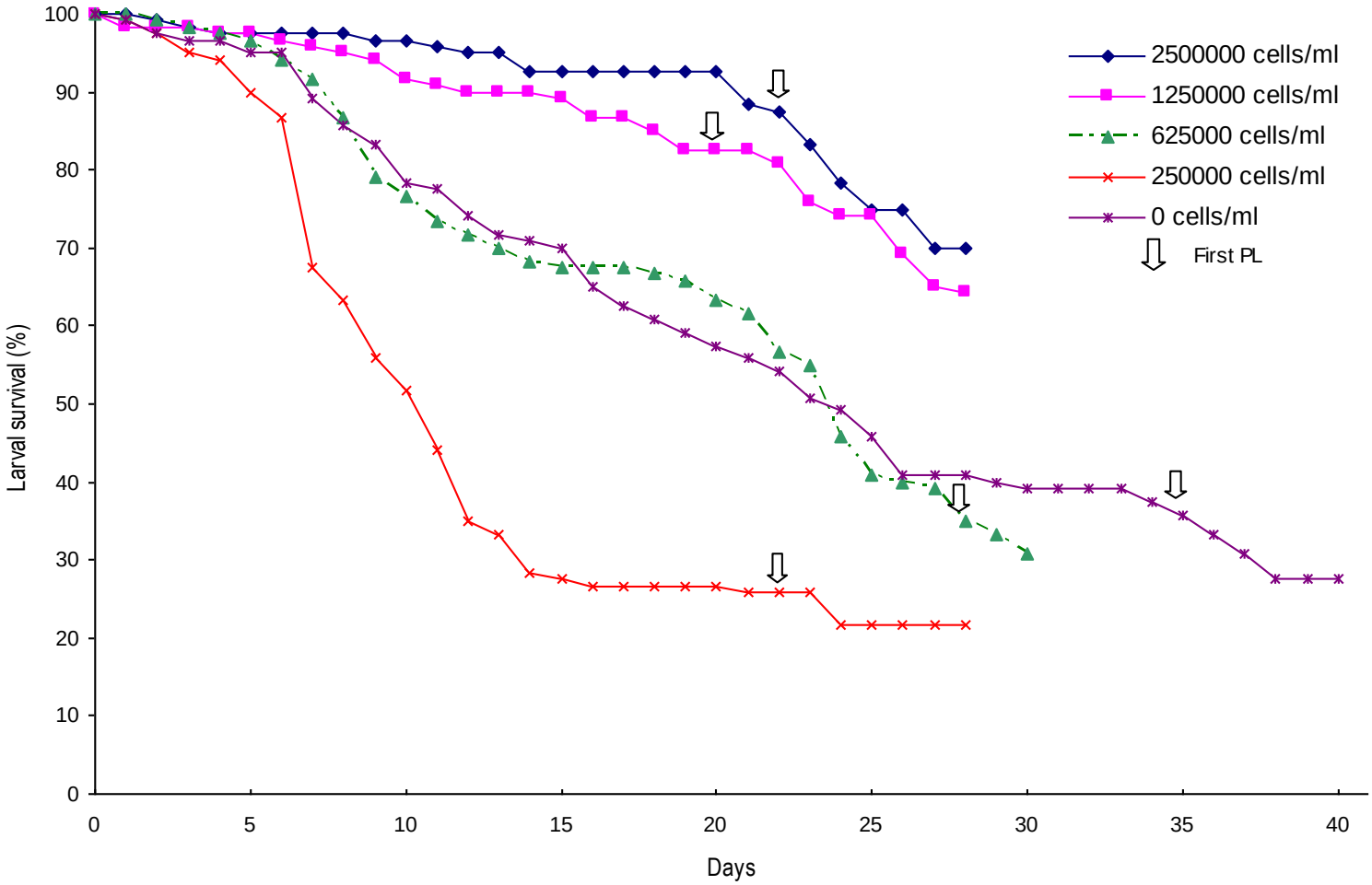
Larval survival (%)
Days
2500000 cells/ml
1250000 cells/ml
625000 cells/ml
250000 cells/ml
0 cells/ml
First PL

Table 6: Mean development time to postlarval stage (PL), carapace length and dry weight of newly settled postlarvae of an Australian Lineage II *Macrobrachium spinipes* cultured at different microalgae (*Nannochloropsis* sp.) concentrations. Data are presented as mean ± S.D. (n=4).

Nannochloropsis sp. concentration (cells/ml)	Mean development time from hatching to PL (days)*	Carapace length of newly settled PL (mm)*	Dry weight of newly settled PL (mg)*
0	**44.3 ± 4.0 ^{c}**	**3.03 ± 0.44 ^{a}**	**0.676 ± 0.177 ^{b}**
2.5 x 10^5	**40.0 ± 5.2 ^{bc}**	**3.03 ± 0.39 ^{a}**	**0.676 ± 0.124 ^{b}**
6.25 x 10^5	**40.1 ± 3.1 ^{bc}**	**2.92 ± 0.31 ^{a}**	**0.704 ± 0.156 ^{ab}**
12.5 x 10^5	**33.2 ± 3.5 ^{ab}**	**3.06 ± 0.20 ^{a}**	**0.852 ± 0.241 ^{a}**
25 x 10^5	**30.6 ± 2.1^{a}**	**3.05 ± 0.25 ^{a}**	**0.852 ± 0.204 ^{a}**

* Different superscripted letters of a same column indicate significant differences ($P < 0.05$). Data are presented as means ± S.D. (n=4).

Table 7: Larval stage index (LSI) for an Australian Lineage II *Macrobrachium spinipes* larvae cultured at different microalgae (*Nannochloropsis* sp.) concentrations.

Nannochloropsis Concentration (cells/ml)	Day 2	Day 5	Day 8	Day 11	Day 14	Day 17	Day 20	Day 23**	Day 26	Day 29	Day32	Day 35	Day 38
0	2.8±0.1^{a}	4.1±0.5^{b}	4.9±0.4^{b}	5.9 ±0.5^{c}	6.4±0.9^{b}	7.1±0.9^{b}	7.7±0.8^{c}	7.9±1.0^{a}	7.9±0.7^{b}	8.7±0.7	8.9±0.4	8.8±0.6	9.5±0.7
2.5 x 10^{5}	2.5±0.7^{a}	4.3±0.6^{b}	5.1±0.5^{ab}	6.2±0.3^{c}	7.5±0.3^{ab}	7.8±1.0^{ab}	8.3±0.5^{bc}	8.9±0.4	9.3±0.2	9.8±0.7	9.9±0.5	10.1±0.3	-
6.25 x 10^{5}	2.5±0.7^{a}	4.8±0.1^{ab}	5.3±0.4^{ab}	6.8±0.4^{bc}	7.4±0.3^{ab}	7.4±0.3^{ab}	7.7±0.5^{c}	8.2±0.7^{a}	8.9±0.4^{a}	8.7±0.4	9.1±0.3	9.5±0.5	-
12.5 x 10^{5}	2.8±0.2^{a}	4.8±0.4^{ab}	5.7±0.1^{ab}	7.3±0.4^{ab}	7.9±0.5^{a}	7.9±0.5^{ab}	9.0±0.5^{ab}	9.2±0.6	9.6±0.3	10.4±0.6	9.9±0.8	-	-
25 x 10^{5}	3.0±0.0^{a}	5.1±0.1^{a}	6.1±0.7^{a}	7.9±0.5^{a}	8.3±0.4^{a}	8.3±0.4^{a}	10.1±0.1^{a}	10.2±0.4	10.4±0.4	10.6±0.3	10.5±0.3	-	-

* Different superscripted letters of a same column indicate significant differences ($P < 0.05$).

** After first appearance of PL, treatments were excluded from the statistical analysis and the data presented after the first PL are for reference only as all PL were removed from culture and not included for the calculation of LSI.

3.5. Discussion

The current study has demonstrated that concentration of *Nannochloropsis* sp. impacts on larval survival, development and growth of the Australian Lineage II of *M. spinipes*. Larval survival to PL stage was significantly higher in the two high algal culture treatments of 12.5 and 25 x 10^5 cells/ ml, suggesting that between algal level of 12.5 x 10^5 cells/ ml and 6.25 x 10^5 cells/ml, a critical threshold may exist (Fig. 7). At the highest algal concentration of 25 x 10^5 cells/ ml, development to PL was the shortest and was significantly faster than in all other treatments except that of the second highest algal density of 12.5 x 10^5 cells/ ml. This was also reflected in LSI values, with in general higher LSI estimates recorded in all high algal concentrations. Significant differences in LSI were registered as early as day 5, suggesting beneficial effects of high algal concentrations being effective earlier during the experiment..

Furthermore, improved larval growth rates in the two high microalgae levels was evident in significantly heavier PL dry weights. It is worth noting that PL carapace length was not significantly different among treatments, suggesting that carapace length is not a good indicator of larval growth rate. Based on the present results, algal culture densities microalgae *Nannochloropsis* sp. for culture of the Lineage II *M. spinipes*, should be added at a level $\geq$ 12.5 x 10^5 cells/ ml.

There are several possible explanations for the apparent beneficial effects of adding high levels of *Nannochloropsis* to larval cultures of the Australian strain of *M. spinipes*. Firstly, addition of *Nannochloropsis* may provide better nutrition to the larvae. Manzi *et al.* (1977) reported that algal cells were found in the gut of larvae of the 'western form' *M. rosenbergii*, however, they were unsure whether the cells had been actively consumed or had been ingested accidentally. These authors further

pointed out that it appeared there was no evidence of any overt assimilation of algal cells or direct nutrition to the larvae (Cohen *et al*. 1976; Joseph, 1977). Judging by the carnivorous nature of *M. spinipes* larvae, the nutritional benefits of direct ingestion of algal cells by larvae is probably limited.

Alternatively, larvae may benefit nutritionally through ingesting *Artemia* grazing on the high abundance of algae. *Artemia* are the major live feed used in culture systems of the 'western form' *M. rosenbergii* larvae. *Artemia* however rare known to lack some essential nutrients, particularly highly unsaturated fatty acids (HUFA), including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), that are important for survival and development of many fish and crustacean larvae (Léger *et al*., 1986). HUFA content of *Artemia* therefore often determines the food value of *Artemia*. Despite lacking DHA, *Nannochloropsis* sp. is known to be rich in EPA (Volkman *et a*, 1989), which may contribute to better nutritional content and quality of *Artemia* as food for freshwater prawn larvae. The extent however, that *Nannochloropsis* sp. contribute to *Artemia* quality is currently unknown because this species is considered an unsuitable diet for *Artemia* nauplii due to toughness and indigestibility of its cell wall (Dhont and Lavens, 1996).

Secondly, the green microalgae *Nannochloropsis* may provide culture conditions that are less stressful for *M. spinipes* larvae and at the same time, may aid their feeding. There is ample evidence to show that underwater lighting conditions and tank colour can play significant roles in foraging success of fish larvae (Ostrowski, 1989; Tamazouzt *et al*, 2002). Less attention has been paid however to crustacean larvae, probably because of the fact that they are capable of feeding in darkness, this response requires a feeding mechanism by either random encounter or chemosensory detection

of prey (Rabbani and Zeng, 2005). Such an ability does not however preclude the possibility that in the presence of light, crustacean larvae may utilise visual cues to enhance their foraging and feeding efficiency (Rabbani and Zeng, 2005). Such a hypothesis is supported by two recent reports that showed tank colour significantly impacted larval survival of the 'western form' *M. rosenbergii* (Yasharian *et al.*, 2005) and the mud crab *Scylla serrata* (Rabbani and Zeng, 2005). Of interest, Yasharian *et al.* (2005) reported that for the 'western form' of *M. rosenbergii*, when the 'clear water' method was adopted, larvae cultured in red and green tanks showed the best and significantly higher survival rates than did larvae reared in white and blue tanks. This agrees with the finding of AQUACOP (1977) who reported best larval survival in dark green tanks. It was suggested that as visual ability and active predatory behaviour increases with development, the ability to effectively pinpoint food items becomes increasingly more important; the green background probably provided better contrast or a silhouette for finding food items, hence resulting in improved larval feeding success, survival rate and growth (Yasharian *et al.*, 2005). The addition of green algae *Nannochloropsis* may have similar effects on larval feeding success as is evident with green coloured tanks. This is also in agreement with the present result that significant improvement in larval performance mainly occurred when higher concentrations of *Nannochloropsis* were added. In a separate study, Lin and Omori (1993) found that feeding rates of larval *M. rosenbergii* decreased with the increase in 'lightness' of the rearing container. They found speed and distance of horizontal movement of the larvae increased by 3 times in white containers compared with those in black containers. Yasharian *et al.* (2005) suggested that such heightened 'excitation' behaviour may contribute to lower feeding rates and that white background may represent a chronic stressor for larval prawns. Similarly, addition of

Nannochloropsis may serve to reduce stress and allow larvae to feed more readily and effectively.

Finally, adding microalgae to larval culture may help improve water quality via reduction of ammonia and other nitrogen wastes in the culture medium (Cohen *et al.*, 1976; New, 2002). However, considering that water used in the current experiment was totally exchanged daily, toxic nitrogen species build up seems less likely to be a major limiting factor. Potentially however microalgae may secrete unknown bioactive chemicals that inhibit various pathogens or may directly benefit the larvae. Obviously, the beneficial effects of adding *Nannochloropsis* to *M. spinipes* larval culture may be multifactorial with additional or synergistic effects. This is an interesting observation that warrants further research. Whatever the underlying mechanisms may be, the outcomes of this study have shown clearly that larvae reared in high microalgae densities performed significantly better. It strongly suggests that microalgae addition is important to larval culture of the Australian Lineage II *M. spinipes* and that a threshold level potentially needs to be reached before such beneficial effects become apparent.

Microalgae have also previously been reported to play a significant role in the success of the larval culture of the 'Western form' *M. rosenbergii*. The pioneer work of Fujimura (1966) and Fujimura and Okamoto (1972) highlighted the importance of using microalgae and *Artemia* nauplii in larviculture as it increased larval survival and reduced the time to reach the PL stage. Other researchers have also confirmed that unialgal supplements, such as *Isochrysis galbana* and *Tetraselmis suecica*, in combination with *Artemia* nauplii can improve larval growth and survival rates (Wickins, 1972). Manzi *et al.* (1977) further reported that algal supplements,

particularly those of the Chrysophta family (i.e. *Isochrysis galbana, Pseudoisochrysis paradoxam* and *Phaedactylum tricornutum*) significantly increased survival and PL production in both static and recirculating culture systems. Unfortunately, the optimal concentration for microalgae was not established experimentally in these studies. For example, algal concentrations trialed ranged from 20,000 – 1,200,000 cells/ ml with an average of 340,000 cells/ ml by Manzi *et al*. (1977). More recently, New (2002) recommended that *Chlorella* sp. to be added in the range of 750,000 – 1,500,000 cells/ ml for green water hatchery culture of 'western form' *M. rosenbergii* larvae. Overall, the ranges of algal addition for 'western form' *M. rosenbergii* larvae culture are in general comparatively higher than those used for marine shrimp, including *Penaeus stylirostris* and *P. vannamei*, of which diatom *Chaetoceros gracilis* concentrations between 30,000 and 100,000 cells/ ml were reported to provide good growth and survival of zoeal larvae (Simon, 1978).

Despite earlier studies reporting showed beneficial effects of the 'green water' culture method for 'western form' *M. rosenbergii* larvae, many commercial hatchery operations have reportedly moved away from 'green water' systems in favour of the 'clear water' method (New, 2002). A possible explanation for this trend is extra requirements needed for facilities, labour and expertise to run for the 'green water' method as well as its management complexity. Another reason as demonstrated here, if algal concentration does not reach a required level, its beneficial effects may be relatively limited, which could well encourage a hatchery manager to abandon the practice. There were however indications that the adaptation of 'green water' culture may be more crucial for the 'eastern form' *M. spinipes* as 'clear water' generally produced < 30 % larval survival for the lineage II larvae in our cultures while substantially higher survival have been reported for the 'western from' *M. rosenbergii*

in ‘clear water’ culture (AQUACOP, 1983), although further assessment is required to justify this. Potentially strain differences may exist, a systematic reassessment of pros and cons of the ‘clear’ vs ‘green water’ method therefore for the hatchery culture of ‘western from’ *M. rosenbergii*, including identifying optimal concentrations, may be worthwhile in the future.

Our results clearly showed that hatchery culture of the Australian Lineage II *M. spinipes* is technically feasible when a ‘green water’ method is employed. The establishment of a ‘green water’ method based on microalgae *Nannochloropsis* sp., a euryhaline, hardy feed species suitable for large scale outdoor culture, could also prove beneficial. Overall, larval survival and development rates of the strain were comparable to those of the ‘western form’. For example, using the ‘green water’ method, Manzi *et al*. (1977) reported survival rates ranging from 75 to 82 % for the ‘western form’ *M. rosenbergii*. First PL’s were observed in our cultures from day 20 to 35 under the various treatments, a result similar, (18 to 35 days) reported for the ‘western form’ (Manzi and Maddox, 1977; Manzi *et al.*, 1977; Malecha *et al.*, 1980). The slightly lower survival rates recorded in the current experiment is most likely due to differences in culture conditions, including use of substantially smaller culture vessels (1 L vs. 60 L) and possibly higher stress levels caused by regular handling during daily water exchange and regular larval staging. In fact, in our larval culture trials using large 400-500 L tanks, survival have been consistently higher than 85 % with PL production as high as 170 PL per litre of culture water achieved. PL’s also appeared as early as day 18 with > 95 % larvae reaching PL stage by day 24-26. The promising larvae culture results evident for the Australian Lineage II *M. spinipes* not only show potential for development of a freshwater prawn aquaculture industry in Australia, but also potential for cross breeding programmes with other strains/ form

(de Bruyn *et al*., 2004b) for the improvement of productivity of freshwater prawn worldwide (New, 2005).

Chapter 4: The effect of *Artemia* enrichment and green water culture on larval survival, development and postlarval fatty acid composition of a native Australian giant freshwater prawn *Macrobrachium spinipes* (Schenkel, 1902).

4.1 Abstract

Newly hatched larvae the of Australian freshwater prawn *Macrobrachium spinipes* were cultured under 6 treatments including both 'greenwater' and 'clearwater' culture conditions using microalgae *Nannochloropsis* sp. at two concentrations and fed with enriched or non-enriched *Artemia* in a 2 x 2 factorial design. Each treatment included 4 replicates where treatments were 1.) 'clearwater' + enriched *Artemia*, 2.) 'clearwater' + unenriched *Artemia*, 3.) 2.5 x 10^6 cells/ ml + enriched *Artemia* 4.) 2.5 x 10^6 cells/ ml + unenriched *Artemia,* 5.) 10 x10^6 cells/ ml + enriched *Artemia* and 6.) 10 x10^6 cells/ ml + unenriched *Artemia.* Larvae were sampled every 3 days to obtain larval stage index (LSI) and after they reached postlarval stage, they were harvested and measured for total length then dried over 24 hours and measured for dry weight. Survival was highest in the 2.5 x 10^6 cells/ ml + enriched *Artemia* treatment (80 %) while the two highest *Nannochloropsis* sp. concentrations of 10 x10^6 cells/ ml of both enriched (74 %) and unenriched (75%) treatments showed similar results and all 'greenwater' treatments were not significantly different from each other. Survival to postlarvae (PL) stage in the 2.5 x 10^6 cells/ ml + unenriched *Artemia* treatment (55 %) were not significantly different from that of the 'clearwater' + enriched *Artemia* treatment (18 %) ($P<0.05$). The 'greenwater' treatments produced the first PL between days 18 – 20 while the 'clearwater' + enriched *Artemia* treatment reached first PL stage on day 23. In contrast, the 'clearwater' + unenriched *Artemia* treatment

resulted in total mortality by day 16. The shortest mean development time to PL was 20.5 and 21.5 days in the 10 x10^6 cells/ ml for enriched and unenriched treatments respectively and both were significantly different ($P<0.05$) from all other treatments, except for 10 x10^6 cells/ ml + unenriched *Artemia* treatment that was not significantly different from 2.5 x 10^6 cells/ ml + enriched *Artemia* treatment. Larval stage index (LSI) for all 'greenwater' treatments showed advanced development in both 'clearwater' treatments except for the 'clearwater' + enriched Artemia was not significantly different ($P<0.05$) from the 2.5 x 10^6 cells/ ml + unenriched *Artemia* treatment. Microalgae density significantly affected mean development time to PL stage while in contrast enrichment did not and no interactions were detected. Larval dry weights of newly settled PL's from all enrichment treatments were heavier than PL's from non-enrichment treatments. The lowest dry weight (0.659 mg) in the 2.5 x 10^6 cells/ ml + unenriched *Artemia* treatment were significantly ($P<0.05$) lower than in all other treatments. Enrichment had a significant effect on dry weight while microalgae density did not. In parallel, total PL length from enrichment treatments were significantly ($P<0.05$) longer than from non-enrichment treatments. The shortest total length from the 2.5 x 10^6 cells/ ml + unenriched *Artemia* treatment was significantly ($P<0.05$) shorter than in all other treatments and no interactions were detected. Enrichment media boosted fatty acid levels including docohexaenoic acid (DHA) 22:6n-3 and eicosapentaenoic acid (EPA) 20:5n-3 in most treatments. DHA in non-enrichment treatments showed decreasing levels with increased microalgae concentration but this trend was not shown with enrichment treatments. DHA levels remained stable at all microalgae concentrations. Linolenic acid (LNA) 18:3n-3 concentration in PL's was not affected by enrichment media while the effect of microalgae was less resolved. While the ratio of n-3/ n-6 was lower in non-enrichment treatments compared with enrichment treatments, ratio's were stable across all

treatments suggesting that *M. spinipes* can adjust the n-3/ n-6 ratio. Effectiveness of *Artemia* enrichment on larval performance was most evident in 'clearwater' cultures with low microalgae concentrations and improved both growth and survival rates. In general, survival in this study was poor, potentially the result of inferior quality of larvae produced from captive bred broodstock. Culture environment with high microalgal densities potentially may provide less stressful light conditions and allow more efficient feeding by larvae. In general, water quality may also be enhanced via algal secretion of bioactive chemicals.

4.2 Introduction

Closure of the life cycle under hatchery conditions of freshwater prawn in Malaysia in the 1960's followed by the development of modern techniques for seed production and commercial farming in Hawaii over subsequent decades has led to wide spread farming of *Macrobrachium rosenbergii* in many countries around the world (New, 2010). While culture now occurs in many parts of the world, the natural distribution of the species is much less limited, and giant freshwater prawn are only found naturally from Pakistan to Vietnam, SE Asia, south to Papua New Guinea, in northern Australia (de Bruyn *et al*., 2004a) and in some Indian and Pacific Ocean Islands (Mather & de Bruyn, 2003). Recent research recognised two genetically distinct forms distributed independently on either side of Huxley's Line, where those found from west of Huxley's barrier were assigned to a 'western' form while those that occurred to the east were defined as the 'eastern' form (de Bruyn *et al*., 2004a). Given this delineation, *M. spinipes* found throughout the tropical northern regions of Australia and endemic to the west of the Great Dividing Range (Short, 2004), belong to the 'eastern' form.

Among Australian *M. spinipes* populations, four genetic lineages were identified, namely Western Australia (lineage I), Gulf of Carpentaria/Northern Territory (lineage II), Irian Jaya (lineage III) and Papua New Guinea/North east Cape York (lineage IV) (de Bruyn *et al*., 2004b). Freshwater prawn aquaculture worldwide is now almost exclusively based on only the 'western' form of giant freshwater prawn, the and recognition of the existence of four lineages of *M. spinipes* in Australia would require independent assessments and development in aquaculture if this species is to be farmed. This is because introduction of any non-native species/strain for aquaculture is illegal in Australia and currently no commercial freshwater prawn farm exist in the country due to earlier failed attempts in to trial culture of local stocks (Lober and Zeng, 2009).

In a recent attempt to re-evaluate aquaculture potential of Australian indigenous freshwater prawn, larvae of Lineage II from the Gulf of Carpentaria were cultured under experimental conditions at James Cook University (JCU) trialling both clear and green water culture methods. Results showed that survival, development and growth of newly hatched larvae to settlement were significantly superior when larvae were cultured using the green water method applying a threshold level of microalgae *Nannochloropsis* sp. concentration and higher of 12.5 x 10^5 cells/ ml. While larval performance was slightly better at 25 x 10^5 cells/ ml differences were not statistically significant when compared with the 12.5 x 10^5 cells/ ml treatment (Lober and Zeng, 2009). This result suggests that it would be worthwhile to investigate whether higher algal concentrations could lead to significant improvement in larval performance.

While addition of microalgae above the threshold level identified in the earlier trial clearly benefited *M. spinipes* larval culture, the underlying mechanisms were not

clear. One possible major contributing factor could be the nutritional benefits obtained by larvae from *Artemia* nauplii grazing on the algae in greenwater systems (Lober and Zeng, 2009). The nutritional role of algae, particularly their essential fatty acid content, has been shown to improve growth performance of larval crustaceans, fish and molluscs in earlier studies (Volkman *et al*., 1989; Duerr *et al*., 1998; D'Souza and Loneragan, 1999). Generally, high concentrations of essential fatty acids, particularly highly unsaturated fatty acids (HUFA), including eicosapentaenoic acid (EPA) 20:5n-3 and docosahexaenoic acid (DHA) 22:6n-3, is indicative of high nutritional value of a microalgae species (Volkman *et al*., 1989). While bivalve larvae normally feed on microalgae directly, larvae and juveniles of most fish and crustaceans feed on zooplankton rather than directly on microalgae in their culture environments. Nutritional benefits of microalgae can still however be obtained indirectly via zooplankton grazing on microalgae that then become the prey of the crustacean larvae (Volkman *et al*., 1989).

While both DHA and EPA have been shown to be vital to survival, development and growth of various crustacean larvae (D'Abramo, 1998), *Artemia* nauplii that is the live prey routinely used for culture of freshwater prawn larvae (New, 2010), is deficient in both essential fatty acids (Léger *et al*., 1986, Watanabe, 1993). In our previous study, *Artemia* used for feeding larvae of freshwater prawn were not enriched (Lober and Zeng, 2009). To evaluate if and to what extent, beneficial effects of green water over clear water culture to the larvae of the Australian strain of freshwater prawn larvae can be attributed to nutritional benefits from *Artemia* grazing on microalgae *Nannochloropsis* sp., tstudy compared the effects of feeding freshwater prawn larvae with both enriched and non-enriched *Artemia* under 'clear water' as well as 'green water' condition. *Nannochloropsis* was maintained at 12.5 x 10^5 cells/ ml.

Meanwhile, a substantially higher microalgae concentration of 10 x 10^5 cells/ ml of *Nannochloropsis* was also used to create 'green water' with larvae fed both enriched and non-enriched *Artemia* to assess if this would further improve larval performance.

4.3. Materials and Methods

4.3.1 *Source of broodstock and larvae*

Broodstock *M. spinipes* were collected from the Flinders River system and its tributaries, near the Gulf of Carpentaria region (latitude 17°52.522; longitude 140°46.837), north Queensland, Australia and were identified as Lineage II from rivers flowing into the Gulf of Carpentaria (Lober and Zeng, 2009). The wild caught broodstock had been utilised for hatchery larval culture in the previous year and postlarvae produced were stocked into outdoor ponds for growout at Walkamin Aquaculture Research Station, Queensland Department of Primary Industries and Fisheries (DPI&F). Large female broodstocks of between 50-70 g were collected from the outdoor ponds and transported to the Marine and Aquaculture Facility Unit (MARFU), James Cook University in Townsville. Berried females were kept individually in 250 L hatching tanks with 5 – 8 psu brackish water and provided with aeration until hatching. Newly hatched larvae were held for 1 h at 12 psu water prior to collection for experimentation.

4.3.2 *Experimental design and set up*

Six larval culture treatments were set up in a two-factor experiment design where *M. spinipes* larvae were fed either enriched or non-enriched *Artemia* under 3 green microalgae concentrations of 0 (clear water), 2.5 and 10 x10^6 cells/ ml. That is, 1) clear water + unenriched *Artemia;* 2) clear water + enriched *Artemia;* 3) 2.5 x 10^6 cells/ ml + unenriched *Artemia';* 4) 2.5 x 10^6 cells/ ml + enriched *Artemia;* 5) 10 x 10^6

cells/ ml + unenriched *Artemia* and 6) 10 x 10^6 cells/ ml + enriched *Artemia*. All treatments were in quadruplicate with 30 newly hatched larvae collected and distributed randomly into a 5 L round container (diameter = 21.5 cm; depth=17.7 cm) as a replicate. Replicates of different treatments were organised in a random block design inside two water baths set at 28 ± 1 °C and with photoperiod set at 14.5L: 9.5D. Culture containers were filled to the top with 12 psu brackish water and closed with lids to prevent larvae jumping out or onto the tank walls. Gentle aeration was provided to each container via a fine-tipped glass pipette inserted through the lid. Larvae in all replicates were cultured from the day of hatching (designated as day 0) until they either reached postlarval stage (PL) or had died. Culture water was exchanged 100 % daily for all replicates in the morning when any mortality was recorded with surviving larvae in each replicate being transferred to an identical container with freshly prepared appropriate food and algal concentrations.

For all treatments and throughout the experiment, larvae were fed a ration of 3 *Artemia*/ ml. Hatching of *Artemia* cysts (INVE Inc, Thailand) was carried out daily in 18 psu water and nauplii hatched were harvested early morning on the following day and divided into two portions with one subset identified for enrichment. Enrichment was carried out using a commercial enrichment emulsion (INVE DC-DHA Selco) prepared according to the manufacturer's instructions and *Artemia* was introduced and enriched overnight before being harvested for feeding larvae on the next day. For the first experimental day, enriched *Artemia* was prepared a day ahead. The gut of *Artemia* were checked under a microscope for presence of enrichment media prior to feeding to larvae.

Microalgae (*Nannochloropsis* sp.) was mass cultured in 3,000 L tanks at JCU's algal culture facility using a commercially available fertiliser (AQUASOL, Yates Ltd, New South Wales, Australia). *Nannochloropsis* cultures were generally re-inoculated every 7-10 days. During the experiment, a stock solution was prepared daily by selecting a *Nannochloropsis* culture at its exponential phase of growth that was then diluted to 12 psu by mixing it with dechlorinated freshwater in a 20 L container with strong aeration. Samples were then taken from the stock solution and algal density assessed using a hemocytometer under a high power microscope. *Nannochloropsis* concentration of the stock solution was estimated by averaging the concentrations of five 1 ml samples. Volume of a stock solution required to prepare a desired concentration of a treatment was calculated using the following equation:

$$C_1V_1 = C_2V_2$$

where C_1 was the designated algal concentration for a particular treatment and V_1 was the total volume required for the treatment for daily water exchange; C_2 was the algal density of the stock solution and V_2 was the volume of the stock solution required to prepare the designated algal concentration for the particular treatment. The required volume of the stock solution was subsequently measured and diluted and adjusted to make up the total volumes required for daily water exchange with a final salinity of 12 psu. Culture water for the clear water treatments (i.e. 0 x 10^6 cells/ ml) was prepared daily by simply adjusting natural seawater to 12 psu with dechlorinated freshwater.

Every 3 days, 16 larvae (4 from each replicate) were sampled randomly from each treatment and their developmental stages identified under a microscope according to Uno and Kwon (1969). Larvae were placed in a small pool of water during the stage identification and returned to the original culture promptly after staging. Such a process has been shown previously not to lead to larval mortality when operated

properly. Once postlarvae were found in any replicate during the daily check, they were removed from the cultures and euthanized by quick freezing. Larvae were then measured for their carapace length (mm) using a microscope equipped with a camera (Leica). They were subsequently dried individually in a 60 °C oven for 24 h before being weighed for dry weight using a Cahn C-33 microbalance (0.001 mg).

4.3.3 Fatty acid analyses of postlarvae

In addition to 4 larval culture replicates in 5 L containers, mass cultures of larvae were carried out separately in 10 L containers under identical culture conditions and daily water exchange regime for each of the six treatments to provide samples of postlarvae for fatty acid analysis. Larval survival and development were not monitored in these cultures but the appearance of postlarvae were checked daily and any newly settled postlarvae was harvested and snap frozen in liquid nitrogen and stored in -80 °C for late fatty acid analyses.

Lipids were extracted from larval samples with chloroform/methanol by the method of Folch *et al.* (1957). Total lipid was determined gravimetrically on an aliquot of the extract by drying for 4 hours at 80 °C in a pre-weighed glass vial. Aliquots of sample lipid extracts were taken for fatty acid analysis. The lipid fatty acids in the extracts were derivitised to their fatty acid methyl esters (FAME) using 14 % boron trifluoride-methanol (Van Wijngaarden, 1967). FAME were analyzed on an Agilent Technologies 6890 gas chromatograph using split injection with helium carrier gas and a flame ionization detector. The column used was a DB23 fused silica capillary column, 30 m x 0.25 mm, with a 0.25 μm coating (Agilent Technologies, USA). Column oven temperature was held at 140 °C for 5 minutes and then elevated at 3 °C/ minute to 210 °C where it was held until all FAME of interest had been eluted. FAME

were identified by comparing their retention times with those of authentic standards (Sigma-Aldrich Co, USA), and were quantified by comparison with the response of an internal standard, heneicosanoic acid.

4.3.4 Data Analyses

Based on results of the larval staging, larval stage index (LSI) was calculated according to Manzi *et al*. (1977) and Mallasen and Valenti (2006):

$$LSI = (\Sigma S_i \times n_i)/N$$

Where, S_i = larval stage (i=1-11; representing each larval stage); n_i = number of larvae in stage S_i; N = total number of larvae examined. The cumulated percentage survival from hatching to postlarval (PL) stage was determined by dividing the total number of successfully metamorphosed PL by the initial number of larvae stocked in each replicate.

Larval stage index (LSI) data were analysed using Two-way ANOVA and Tukey's test was used to detect significant differences between treatments at the 0.05 level of significance. However, for those data that did not meet the assumptions of ANOVA, i.e. cumulated larval survival from hatching to PL, mean larval development time, dry weight and total length of newly settled PL were analysed using Kruskal-Wallis non-parametric test and significant effects between factors were assessed using a Mann-Whitney test. All data was analysed using statistics package, SPSS Version 16.0.

4.4 Results

4.4.1 Larval survival

Figure 1 shows cumulative larval survival from newly hatched larvae to PL stage in all five treatments except for the 'clear water + unenriched *Artemia'* treatment that suffered 100 % mortality. Under 'clear water' culture conditions, no larva survived to PL stage when fed unenriched *Artemia*.In the treatment fed with enriched *Artemia* however, a survival rate of 18.0 % to PL stage was achieved for the 'clear water + enriched *Artemia*' treatment (Fig. 9). In contrast, in all 'green water' treatments with *Nannochloropsis* added at both levels of 2.5 and 10 x 10^6 cells/ ml, larval survival were substantially improved to between 55.8 % and 80.0 %. At lower *Nannochloropsis* concentrations of 2.5 x 10^6 cells/ ml, survival to PL stage for larvae fed enriched *Artemia* was also higher (80.0 %) than those fed non-enriched *Artemia* (55.8 %). No significant differences were detected however between the two treatments. When compared to the 'clear water + enriched *Artemia*' treatment however, larval survival in the '2.5 x 10^6 cells/ ml + enriched *Artemia'* treatment was significantly higher, but this was not the case for the '2.5 x 10^6 cells/ ml + non-enriched *Artemia'* treatment (Fig. 9). At a higher *Nannochloropsis* level of 10 x 10^6 cells/ ml, larval survival of 'enriched' and 'non-enrichment' *Artemia* treatments (74 vs 75 %) were very similar and both were significantly above that of the 'clear water + enriched *Artemia*' treatment (Fig. 9).

Daily larval survival rates in all treatments is presented in Figure 10. Results show that in all treatments, larval mortality was very low over the first week with survival rates maintained at between 95 -100 %. From day 9 onward however, larval survival in both 'clear water' treatments started to decline considerably and 'clear water + non-enriched *Artemia'* treatment reached total mortality by day 16 (Fig. 10). Larval survival in the 'clear water + enriched *Artemia'* treatment fared better with survival rate stabilising after day 17 at a low level and with the first PL appearing on Day 23.

In contrast, whether larvae were fed enriched or non-enriched *Artemia,* daily survival in all 'green water' treatments remained high and only declined gradually with the first PL appearing several days earlier than in any clearwater treatment between Day 18 to 20. It is worth noting that in the '2.5 x 10^6 cells/ ml + non-enriched *Artemia'* treatment, a single larva survived for a prolonged period without moulting to PL and eventually died on Day 54 (Fig. 10).

4.4.2 Larval development

There was a general trend of shorter larval development times to PL stage with increasing microalgae concentration while any effects of *Artemia* enrichment appeared to be only relatively limited (Tab. 8). Statistical analysis confirmed that microalgae concentration had a significant effect on larval development time to PL stage ($p<0.05$) while *Artemia* enrichment did not and no interactions was detected between the two factors.

Mean development times to PL stage were the shortest in the highest microalgae concentration of 10 x 10^6 cells/ ml with *Artemia* enrichment treatment (20.5 days) and only slightly shorter in the non-enrichment (21.5 days) treatment. Both treatments showed significantly shorter development times than in all other treatments except for that of the '10 x 10^6 cells/ ml + non-enrichment *Artemia'* treatment was not significantly different from the '2.5 x 10^6 cells/ ml + enriched *Artemia'* treatment (25.8 days). The longest mean development time was recorded for the 'clear water + enriched *Artemia'* treatment (27.3 days) but this was excluded in the statistical analysis due to low number of larvae attaining the PL stage in this treatment.

Further examination of LSI data demonstrated that differences in larval development rate were evident as early as Day 3 in larval culture (Tab. 9). On day 3, LSI of larvae reared under 'green water' conditions with *Nannochloropsis* added at 10 or 2.5 x 10^6 cells/ ml were significantly different ($P<0.05$) and showed more advanced development compared with both 'clear water' treatments, except that the treatment of 'clear water + enriched *Artemia*' was not significantly different from that of the '2.5 x 10^6 cells/ ml + non-enrichment *Artemia'* treatment (Tab. 9). Over the subsequent culture period, the 'green water' treatments consistently showed higher LSI values compared with 'clear water' treatments. Under the same 'clear water' conditions, 'enrichment' treatments showed significantly higher LSI values than 'non-enrichment' treatments from Day 9 onward (Tab. 9).

4.4.3 Postlarval dry weight and total length

Dry weights (DW) of newly settled PL's in 'enrichment' treatments were heavier than PL's of 'non-enrichment' treatments (Tab. 8). Statistical analysis confirmed that *Artemia* enrichment significantly increased PL dry weight while microalgae concentration did not ($P<0.05$). The lowest dry weight (0.659 mg) was recorded for the 'non-enrichment *Artemia'* treatment at the lowest algal density of 2.5 x 10^6 cells/ ml, which was significantly lower than in all other treatments ($p<0.05$).

PL total length, in general showed a similar trend as for dry weight with enriched treatments resulting in longer total lengths than those evident in non-enrichment treatments (Tab. 8). The shortest total length registered for the non-enrichment *Artemia'* treatment at lower algae density of 2.5 x 10^6 cells/ ml was also significantly lower than in all other treatments ($p <0.05$). No significant differences were detected among 'enrichment' treatments (Tab. 8).

4.4.4 Fatty acid profile of postlarvae

Although no larvae reached PL stage in (30 larvae per replicate) the 'clear water + non-enrichment *Artemia'* treatment, some larvae did manage to reach PL under mass culture in this culture condition which allowed fatty acid analysis to be conducted.

Results of fatty acid analysis of newly settled PL's (Table 10) showed clear evidence that *Artemia* enrichment boosted larval fatty acid content (mg fatty acid/ g larval dry weight) in all fatty acid groups, including saturated fatty acids (∑SFA), monounsaturated fatty acids (∑MUFA), polyunsaturated fatty acids (∑PUFA) and highly unsaturated fatty acids (∑HUFA) and total fatty acids. Postlarvae in all enriched *Artemia* treatments had substantially higher total fatty acid contents (69.91-77.18 mg/ g) than those from non-enriched *Artemia* treatments (ranging from 47.28 - 55.31 mg/ g). Similar results were found for ∑SFA, ∑MUFA, ∑PUFA and in particular for ∑HUFA, in all enriched treatments that were on average more than twice that of any non-enrichment treatments (Tab. 10). In contrast, effects of microalgal concentration were limited and varied within treatment (Tab. 10).

Among HUFAs that are considered to be most important for larval quality, i.e. Docohexaenoic acid (22:6n-3, DHA), Eicosapentaenoic acid (20:5n-3, EPA) and Arachidonic acid (20:4n-6, ARA), levels were substantially boosted in all enriched treatments, and were consistently higher than in any non-enrichedtreatments (Tab. 10). In particular, DHA had increased by more than 4, 11 and 16 times, respectively under 'clear water 0 x 10^6 cells/ ml', 2.5 x 10^6 cells/ ml and 10 x 10^6 cells/ ml culture conditions. This in contrast resulted in an interesting trend so that when *Artemia* were not enriched, DHA levels actually declined with increased microalgae concentration.

The same trend however, was absent in enriched treatments and DHA levels remained effectively stable at different algal concentrations (Tab. 10). For EPA and ARA, a similar pattern was observed although the effect was reduced and DHA levels were generally lower than for EPA (Tab. 10). For the important PUFA, linoleic acid (18:2n-6, LOA) content was also substantially higher in all enriched treatments; although, linolenic acid (LNA, 18:3n-3) was an exception as it was not boosted by enrichment (Tab. 10). The effects of algal concentration on LOA and LNA were less clear and were divergent from that of HUFAs (Tab. 10)

Of interest, while n-3/ n-6 ratios were consistently lower for non-enriched treatments when compared with enriched treatments, ratio's were very stable among all non-enriched treatments (ranging from 1.22-1.26) as well as enriched treatments (between 1.38-1.40). This suggests that giant freshwater prawn larvae appear to show a strong capacity to adjust n-3/ n-6 ratio. This potentially suggests that maintaining this ratio is important.

Figure Legends

Figure 9: Cumulative larval survival from hatching to postlarvae of an Australian freshwater prawn *Macrobrachium spinipes,* cultured at different concentrations of *Nannochloropsis* sp. and fed either enriched or non-enriched *Artemia* respectively. Data are presented as mean ± SD (n=4). Different superscripted letters indicate significant differences ($P < 0.05$).

Figure 10: Daily percentage survival of larvae of an Australian giant freshwater prawn *Macrobrachium spinipes,* cultured at different concentrations of *Nannochloropsis* sp. and fed either enriched or non-enriched *Artemia,* respectively. Note that postlarvae survival were not included and the survival line of a treatment terminated on the day all larvae in the treatment became PL or dead**.**

Figure 9

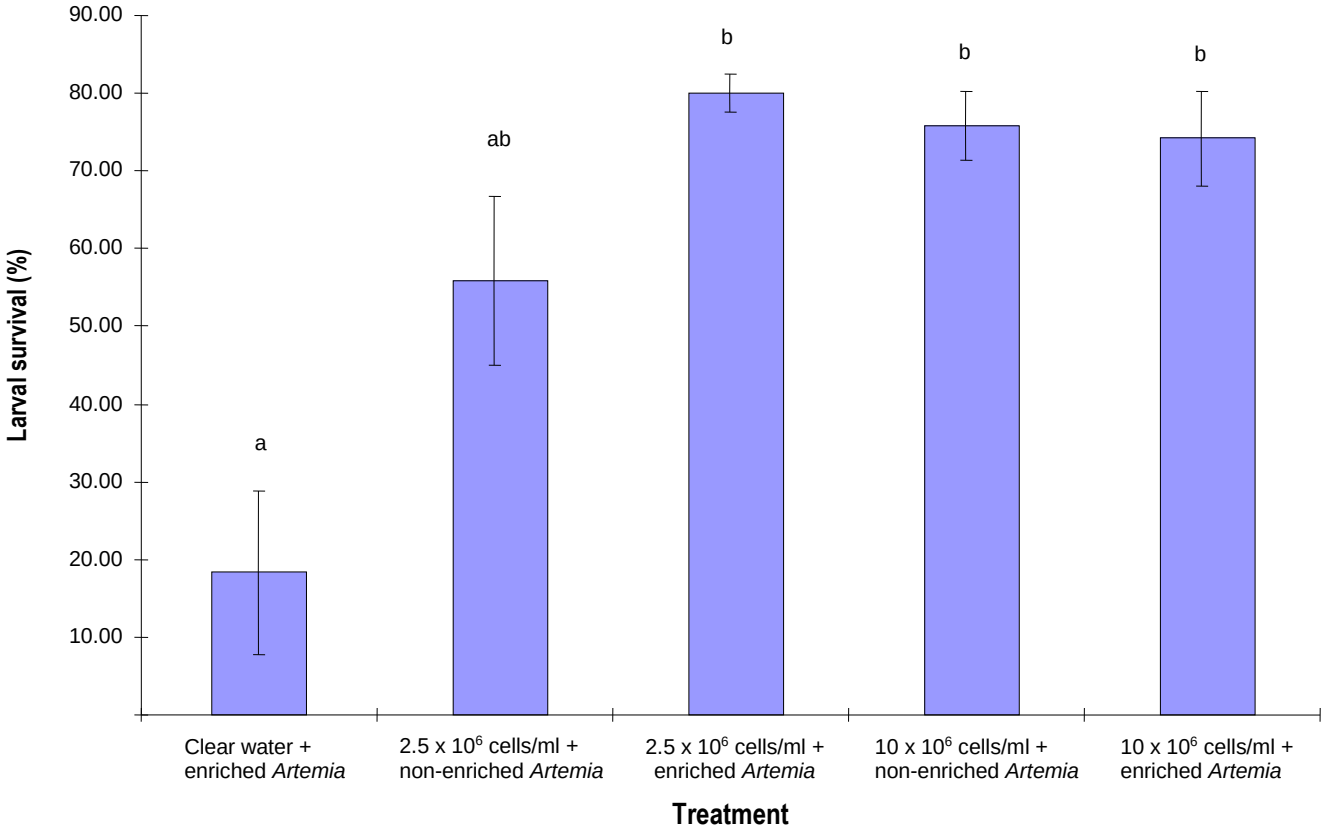
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
Larval survival (%)
a
ab
b
b
b
Clear water + enriched Artemia
2.5 x 10^6 cells/ml + non-enriched Artemia
2.5 x 10^6 cells/ml + enriched Artemia
10 x 10^6 cells/ml + non-enriched Artemia
10 x 10^6 cells/ml + enriched Artemia
Treatment

Figure 10

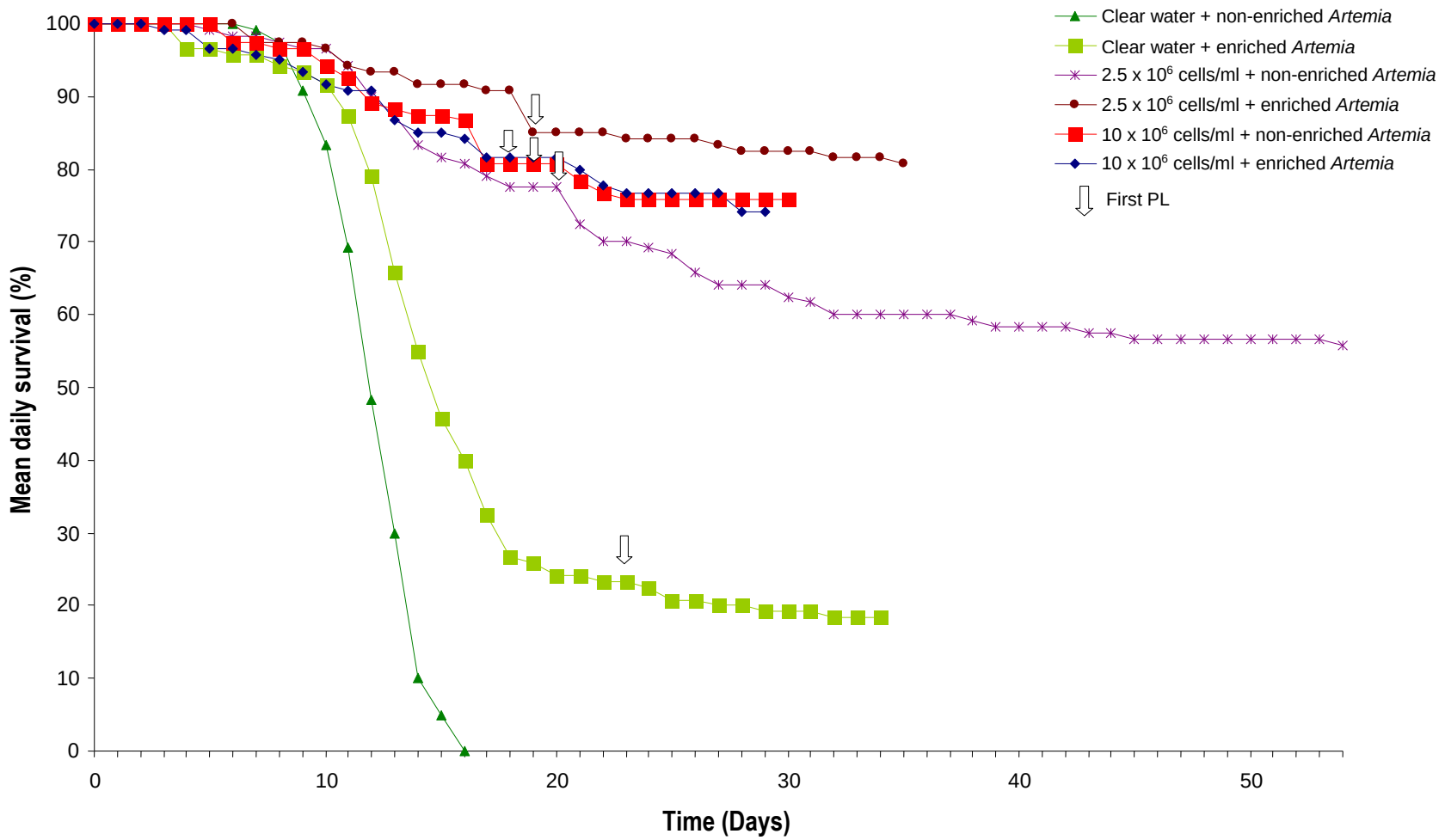
Clear water + non-enriched Artemia
Clear water + enriched Artemia
2.5 x 10^6 cells/ml + non-enriched Artemia
2.5 x 10^6 cells/ml + enriched Artemia
10 x 10^6 cells/ml + non-enriched Artemia
10 x 10^6 cells/ml + enriched Artemia
First PL
Mean daily survival (%)
Time (Days)
0
10
20
30
40
50
60
70
80
90
100

Table 8: Mean larval development time, dry weights and total lengths of newly settled postlarvae of Australian *Macrobrachium spinipes* postlarvae cultured at different concentrations of *Nannochloropsis* sp. and fed either enriched and non-enriched *Artemia*

Treatment	Mean development time (days)*	Dry weight (mg)*	Total length (mm)*
$10x10^6$ cells/ml + enrichment *Artemia*	20.5 ± 0.2 [a]	0.863 ± 0.033 [a]	10.63 ± 0.25 [ab]
$10x10^6$ cells/ml + non-enriched *Artemia*	21.5 ± 1.6 [ac]	0.848 ± 0.029 [a]	10.46 ± 0.06 [a]
$2.5x10^6$ cells/ml + enrichment *Artemia*	25.8 ± 2.4 [b]	0.928 ± 0.019 [b]	10.80 ± 0.22 [b]
$2.5x10^6$ cells/ml + non-enriched *Artemia*	27.0 ± 4.0 [bc]	0.659 ± 0.044 [c]	9.92 ± 0.42 [c]
Clear water + enriched *Artemia*	27.3 ± 1.3	0.934 ± 0.228	10.56 ± 0.98
Clear water + non-enriched *Artemia*	-**	-	-

*Different superscripts in a column indicate significant differences ($p<0.05$); data are presented as mean ± SD.

**data unavailable under 'clear water' condition due to total larval mortality.

Table 9: Larval stage index (LSI) of Australian *Macrobrachium spinipes* larvae cultured at different concentrations of *Nannochloropsis* sp. and fed either enriched or non-enriched *Artemia*

Treatment	Day 3	Day 6	Day 9	Day 12	Day 15*	Day 18*
Clear water + non-enriched *Artemia*	2.5 ± 0.2 [a]	4.7 ± 0.4 [a]	4.9 ± 0.1 [a]	4.8 ± 0.4 [a]	5.4 ± 0.4	-
Clear water + enriched *Artemia*	2.6 ± 0.1 [a]	4.8 ± 0.3 [a]	5.8 ± 0.6 [b]	6.4 ± 1.0 [b]	6.8 ± 2.4	6.0 ± 4.4
$2.5x10^6$ cells/ml + non-enriched *Artemia*	2.9 ± 0.3 [ab]	5.0 ± 0.2 [a]	6.0 ± 0.4 [b]	6.6 ± 0.9 [b]	8.3 ± 1.0 [b]	8.1 ± 0.9 [a]
$2.5x10^6$ cells/ml + enriched *Artemia*	2.9 ± 0.1 [b]	5.1 ± 0.3 [a]	5.9 ± 0.8 [b]	7.0 ± 1.3 [b]	8.9 ± 1.3 [b]	8.9 ± 1.2 [a]
$10x10^6$ cells/ml + non-enriched *Artemia*	3.2 ± 0.2 [b]	5.1 ± 0.1 [a]	5.9 ± 0.1 [b]	7.4 ± 0.5 [b]	8.4 ± 1.1 [b]	9.2 ± 1.1 [a]
$10x10^6$ cells/ml + enriched *Artemia*	3.2 ± 0.4 [b]	5.1 ± 0.3 [a]	6.2 ± 0.6 [b]	7.0 ± 0.2 [b]	9.4 ± 0.9 [b]	10.7 ± 0.2 [b]

*Different superscripts in a column indicate significant differences ($p<0.05$); data are presented as mean ± SD.
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Table 10: Fatty acid profile (mg fatty acid/g larval dry weight) of Australian *Macrobrachium spinipes* postlarvae cultured at different concentrations of *Nannochloropsis* sp. and fed either enriched or non-enriched *Artemia*

	TREATMENT					
Fatty Acid (mg/g of dry sample)	Clear water + non-enriched *Artemia*	Clear water + enriched *Artemia*	2.5×10^6 cells/ml + non-enriched *Artemia*	2.5×10^6 cells/ml + enriched *Artemia*	10×10^6 cells/ml+ non-enriched *Artemia*	10×10^6 cells/ml + enriched *Artemia*
14	0.63	0.97	0.66	0.90	0.60	1.18
15	0.16	0.28	0.15	0.25	0.12	0.34
16	7.84	11.85	8.27	10.73	8.50	11.90
17	0.49	0.78	0.45	0.69	0.52	0.71
18	5.40	7.64	5.27	6.90	6.52	5.60
19	-	-	-	-	-	-
20	0.14	0.18	0.16	0.16	0.16	0.21
22	0.14	0.17	0.19	0.16	0.16	0.33
24	0.15	0.12	0.18	0.12	0.16	0.25
ΣSFA	**14.95**	**22.01**	**15.33**	**19.92**	**16.75**	**20.54**
14:1n-5	-	-	-	-	-	-
16:1n-7	0.35	1.04	0.57	1.01	0.54	1.35
17:1n-8	-	-	-	-	-	-
18:1n-9	8.57	16.26	9.58	14.63	10.17	15.50
18:1n-7	3.14	5.18	3.07	4.66	3.78	4.26
20:1n-11	-	0.09	-	0.08	-	0.16
20:1n-9	0.26	0.87	0.31	0.80	0.40	0.87
20:1n-7	-	-	-	-	-	-
22:1n-11	-	-	-	-	-	-
22:1n-9	-	-	-	-	-	-
22:1n-7	-	-	-	-	-	-
24:1n-9	-	-	-	-	-	0.10
ΣMUFA	**12.33**	**23.44**	**13.54**	**21.17**	**14.89**	**22.24**
18:2n-6 (LOA)	2.65	6.17	3.16	5.49	3.14	5.28
18:3n-3 (LNA)	9.11	8.24	12.04	7.58	12.96	6.55
18:4n-3	0.71	0.54	1.19	0.51	1.37	0.48
20:2n-6	0.25	0.82	0.24	0.75	0.32	0.50
20:3n-6	-	-	-	-	-	-
20:4n-6 (ARA)	1.10	1.72	1.01	1.62	0.86	1.66
20:3n-3	0.99	1.60	1.17	1.55	1.81	0.72
20:4n-3	-	-	-	-	-	-
20:5n-3 (EPA)	3.92	6.87	3.61	6.40	2.82	6.15
22:4n-6	-	-	-	-	-	-
22:3n-3	-	-	-	-	-	-
22:5n-6	0.09	0.24	0.08	0.23	-	0.28
22:5n-3	0.10	0.24	0.10	0.22	0.08	0.32
22:6n-3 (DHA)	1.09	5.29	0.43	5.08	0.32	5.20

ΣPUFA	**20.00**	**31.73**	**23.04**	**29.42**	**23.68**	**27.14**
n-3 PUFA	15.91	22.77	18.55	21.33	19.36	19.42
n-6 PUFA	4.09	8.96	4.50	8.09	4.32	7.72
n-3/n-6	1.26	1.39	1.24	1.38	1.22	1.40
ΣHUFA	**7.54**	**16.78**	**6.65**	**15.84**	**6.21**	**14.82**
TOTAL	**47.28**	**77.18**	**51.92**	**70.51**	**55.31**	**69.91**

"-" indicates <0.05mg/g

4.5 Discussion

The results of the present study confirm the hypothesis resulting from a previous study (Lober and Zeng, 2009) that compared with the 'clear water' method, 'green water' culture conditions can significantly improve larval performance of Lineage II Australian freshwater prawn *M. spinipes*. While earlier research had demonstrated a threshold concentration of microalgae *Nannochloropsis* (1.25 $x10^6$ cells/ml) was required to achieve high larval survival rates (Lober and Zeng, 2009), results of the current study showed that a very high *Nannochloropsis* concentration of 10 $x10^6$ cells/ml significantly enhanced larval development when compared to 2.5 $x10^6$ cells/ml. This implies that under 'green water' culture conditions for *M. spinipes* larvae, attention should be paid to maintaining a threshold level of *Nannochloropsis* in the culture water, and, when water condition permits, *Nannochloropsis* can be added to very high levels to further benefit larval development rates.

While *Artemia* enrichment in contrast has also been shown to improve larval survival rates effects appear to decline with increasing *Nannochloropsis* concentration (Fig. 1). Under 'clear water' culture conditions, larvae achieved 18% survival when fed enriched *Artemia* while total mortality was experienced when they were fed unenriched *Artemia.* At the lower *Nannochloropsis* concentration of 2.5 $x10^6$ cells/ml, larval survival was also higher with *Artemia* enrichment. Although the difference was not statistically significant between the enriched and non-enriched treatments, the enriched treatment showed significantly higher survival rates than did the 'clear water + enriched *Artemia*' treatment while the non-enriched treatment was not affected. When *Nannochloropsis* concentration was increased further to10 $x10^6$ cells/ml however, larval survival rate became very similar for enriched and non-enriched treatments. Results suggest therefore that

Artemia enrichment is more crucial under 'clear water' conditions or at low algal concentrations in greenwater systems.

It is worth noting that under identical culture conditions, larval survival was substantially poorer in the present study in comparison with the previous experiment (Lober and Zeng, 2009). For example, when cultured under the same 'clear water + non-enriched *Artemia*' conditions, larvae suffered total mortality in the present study but had showed 30% survival in the previous study. Similarly, 55.8% survival was recorded for '2.5 x 10^6 cells/ ml + non-enriched *Artemia'* treatment in the present experiment, while it reached 70.8% in the previous study (Lober and Zeng, 2009). These results suggest that larval quality in the current experiment may have been inferior and this probably could be attributed to the fact that broodstock prawn used in the present experiment were captive bred from a same batch of larvae while those used in the previous study were wild caught with high genetic diversity. In-breeding is likely to reduce genetic diversity and leads to poor quality larvae. Furthermore, the total mortality suffered from the 'clear water + non-enriched *Artemia*' treatment in the present study suggests that when larval quality is low, providing 'green water' culture condition becomes more crucial.

Larval development appeared to be mainly affected by concentration of algae in the culture vessel while *Artemia* enrichment boosted growth rate of larvae. Larval development was more advanced at high microalgae concentration of 10 x 10^6 cells/ml (20.5 and 21.5 days in both enriched and non-enriched treatments respectively) compared with lower microalgae concentrations (25.8 - 27.3 days). Larval stage index (LSI) data further supports the contention that development rate was more rapid and was achieved as early as day 3 in high density microalgae cultures (10 x 10^6 cells/ ml). While there was also some suggestion that *Artemia* enrichment may improve larval

development, any effects were clearly much more limited. In contrast, postlarvae were heavier and larger in all treatments fed enriched *Artemia* than those fed non-enriched *Artemia*, suggesting major beneficial effects on larval growth with *Artemia* enrichment. It should be noted that any growth promoting effects of *Artemia* enrichment appeared to be much more evident at low microalgae density of 2.5 x 10^6 cells/ ml.

As expected, higher contents (mg/ g) of fatty acids were recorded in the newly settled PL's in all *Artemia* enriched treatments. This result was evident across all fatty acid groups and most essential fatty acids, including DHA, EPA, ARA and LOA all showed significantly higher concentrations in treatments where larvae were fed enriched *Artemia*. DHA levels, in particular, were 4-16 times higher in enriched treatments than that in non-enriched treatments at the same algal concentration. Substantially higher fatty acid content, particularly essential fatty acids in postlarvae indicates the beneficial effects of *Artemia* enrichment and demonstrates that the enrichment emulsion used and the procedure performed were both effective. In general, *Nannochloropsis* concentration appeared to have major effects on fatty acid content of PL's even though DHA content decreased with increased *Nannochloropsis* concentration. This potentially may result from the fact that the microalga, *Nannochloropsis* is known naturally to contain very low levels of DHA (Volkman *et al.*, 1989).

The current study is the first that has investigated the effects of varying levels of microalgae and *Artemia* enrichment on growth and survival rates on freshwater prawn *M. spinipes* in larval culture. In fact, to date there have been very few studies that have examined the effects of microalgae levels or ration and type of algal diets on larvae culture performance in crustaceans (D'Souza & Loneragan, 1999; Piña *et al*., 2006). Summarising results from a variety of larval

performance indices, including survival, development and growth rates, *Artemia* enrichment appears to be most beneficial under 'clear water' culture conditions but any effects appear to diminish with increasing algal (*Nannochloropsis*) concentration. At a high microalgae concentration of 10 x 10^6 cells/ml *Artemia* enrichment was shown to only slightly enhance larval development and growth rates, but any differences were not statistically significant. Hence, enrichment of live feeds may not be necessary at high microalgae concentrations as larvae appear capable of relying solely on benefits derived from a dense microalgal environment to achieve best performance. Mechanisms underlying the beneficial effects generated by a dense microalgae culture environment remain however unclear. A review of available literature, suggests three possible explanations (Lober and Zeng, 2009): 1) nutritional benefits of larvae are gained from directly feeding on algae or indirectly by ingesting *Artemia* grazing on *Nannochloropsis* (Manzi & Maddox, 1977); 2) Dense microalgae possibly provide less stressful underwater light conditions as well as backdrop for more efficient capture and feeding of *Artemia* by larvae (Lin and Omori; 1997; Yasharian *et al*., 2005; Rabbani and Zeng, 2005) and 3) Improved water quality induced by algal secretion of bioactive chemicals that inhibit pathogens/benefit larvae that also reduces nitrogen wastes in culture water as a result of algae photosynthesis (Cohen, *et al*., 1976). Postlarval fatty acid analysis results showed however, that larvae fed non-enriched *Artemia* under both 'clear water' and 'green water' conditions were similar, and this would suggest that the nutritional benefit hypothesis is probably not a major contributing factor, which is consistent with the suggestion that *Nannochloropsis* is not considered in general to be a suitable diet for *Artemia* nauplii due to the relative toughness and indigestibility of its cell wall (Dhont and Lavens, 1996). This leaves the other two possibilities or possible synergistic effects of various mechanisms as more likely explanations. This very interesting suggestion warrants further research.

Chapter 5. Effect of stocking density and shelter on survival and growth of newly settled postlarvae of Australian giant freshwater prawn *Macrobrachium spinipes* (Schenkel, 1902).

5.1 Abstract

Newly settled postlarvae of Australian native *M. spinipes* were cultured under different initial stocking densities of 500 (low), 1000 (intermediate), 2000 PL m^{-3} (high) with or without shelter respectively, to test for effects of stocking density and shelter availability and their potential interactions on relative performance of nursery culture of prawn postlarvae. For each treatment (n = 6), four replicates were employed and the trial lasted for 60 days. Statistical analysis detected no significant interaction ($P>0.05$) between stocking density and shelter on any measured variable, so the main effects were analysed separately. Firstly, for survival rate results in the two same density treatments (i.e. with and without shelter) pooled, the low stocking rate (500 PL m^{-3}) produced significantly higher mean survival (84.8 %) compared with the high density treatment (2000 PL m^{-3}) (57.4 %) ($P<0.05$). The intermediate density (1000 PL m^{-3}) was intermediate (74.4 %), but significantly different ($P<0.05$) from both the high and low density treatments. At the end of the trial, the high stocking density produced significantly heavier juveniles (wet weight: 144.24 ± 0.01 mg ind^{-1}), that was almost 3 times higher in final biomass (13.45 ± 1.39 g) and an increase in biomass (8.87 ± 1.28 g), than was evident in the low density treatment (wet weight: 117.99 ± 0.003 mg ind^{-1}). The 2000 PL m^{-3} treatment also resulted in significantly higher individual wet weight and final biomass than was evident in the medium density of 1000 PL m^{-3} (wet weight: 124.61 ± 0.003 mg ind^{-1}; final biomass: 8.22 ± 0.46 g). In contrast, addition of shelter produced significantly heavier mean juvenile wet weight (134 ± 0.01 vs. 123.61 ± 0.003 mg ind^{-1}) and higher

final biomass (11.76 ± 1.84 vs. 8.79 ± 1.12 g) at harvest as compared with no shelter. Given these results, the best production can be achieved wither with high stocking density of 2000 PL m^{-3} (when the final production is the main aim) or a medium density of 1000 PL m^{-3} (when PL survival is the main concern) over the nursery culture phase of *M. spinipes* postlarvae. Addition of shelter also further enhances both mean wet weight of juveniles and final biomass.

5.2. Introduction

Since the life cycle of the giant freshwater prawn *Macrobrachium rosenbergii* was first closed in Malaysia (Ling, 1969), efforts to further develop and improve modern commercial culture practices for this species have been ongoing (Fujimura and Okamoto, 1972; New, 2005). As a result, farming of the *M. rosenbergii* has continued to expand worldwide (New, 2009). Even though giant freshwater prawn is native only to tropical and sub-tropical regions across the Indo-Pacific region (Mather and de Bruyn, 2003), over the past decades, this species has been introduced extensively outside of its natural distribution range for aquaculture purposes (New, 2002). Recent research on wild stocks of the species have recognised two genetically divergent clades referred to as 'eastern' and 'western' forms. The 'eastern form' is restricted to the east of Huxley's Line, a biogeographical barrier that divides the Indonesian archipelago north to the Philippines. The eastern form of *M. rosenbergii* is mainly found in Australia and Papua New Guinea, while the 'western form' that occurs west of Huxley's Line, is present in South and South East Asia (de Bruyn *et al*. 2004a). All Australian giant freshwater prawn populations conform to the 'eastern form' which was recently renamed *M. spinipes* after a formal nomenclature revision (Ng &Wowor, 2011). A recent molecular analysis, *M. spinipes* across Australia recognised four lineages (de Bruyn *et al*. 2004b). Since worldwide development of the giant freshwater prawn

aquaculture industry over the last century was largely based on wild broodstock collected from Asia, technical information about culture practices have almost exclusively been derived from research conducted on the western species (*M. rosenbergii*). This form has accounted for virtually all of the world wild fisheries and aquaculture production of giant freshwater prawn (New, 2010).

Based on culture practices developed for growout of *M. rosenbergii* in tropical regions, culture can be year round (Rao, 1991). In temperate and sub-tropical regions however, low temperatures limit production to only the warmer months (Tidwell and D'Abramo, 2010). As a consequence, nursery culture (and overwintering of broodstock) of *M. rosenbergii* are often required in locations with climatic and/or water restrictions (New 1990). An intermediate phase between hatchery and pond stocking of juveniles, can improve productivity and profitability in colder environments (Sandifer and Smith, 1977; Cohen and Barnes, 1982). In addition, holding *M. rosenbergii* postlarvae in nurseries also allow for substantially better control and management of culture practices, including feed administration, water quality management and predator control (Alston, 1989). This can significantly improve production and value at harvest as this will often lead to production of larger mean sized prawns (Sandifer and Smith, 1977; Ra'anan and Cohen, 1982). Nurseries are now often applied in the tropics (New, 2002).

When larvae are stocked at relatively high densities in nurseries, aggressive behaviour and the natural cannibalistic tendency of *M. rosenbergii* juveniles is intensified, particularly when individuals moult and immediately after a moult when animals are still soft-shelled (Sandifer and Smith, 1977). This has the potential to substantially impact on survival and growth of juvenile prawns. Hence, identifying the highest stocking density that maximises productivity (survival and growth rate) is crucial for nursery culture (Siddiqui *et al*., 1997; Coyle *et al*., 2003). At the same

time, addition of substrate, whether natural or artificial, is now a common practice in *M. rosenbergii* nursery culture to improve survival (New, 2002). Availability of substrate as refuges for young prawns has been considered to be a way to reduce impacts of aggressive behaviour and cannibalism, particularly when juveniles are most vulnerable, hence leading to higher overall productivity (Sandifer and Smith, 1977; Cohen *et al*., 1983; Tidwell *et al*., 1998).

Past attempts at culturing the native giant freshwater prawn (*M. spinipes*) in Australia to date have not been successful, reportedly due to a lack of technical expertise and infrastructure, low hatchery survival and high cannibalism rates (Cantrelle, 1988), complicated further by disease problems (Bergin 1986; Owens and Evans 1989). With the recent identification of the existence of four distinct genetic strains of *M. spinipes* in Australia (de Bruyn *et al*. 2004b), interest has been revived in potential for farming here. As hatchery culture trials of Lineage II *M. spinipes* showed promising results (see Chap. 3 and 4), the next stage will be to examine their growout potential. Our previous investigation showed that the natural spawning season of Australian *M. spinipes* ranged from late November to March and unlike *M. rosenbergii* this species is apparently difficult to induce to spawn outside of the natural breeding season even via temperature and photoperiod manipulation (Chap. 2). As tropical North Queensland fresh water temperatures often can drop below 20 °C during the coldest annual months from May to August in the southern hemisphere, water temperatures are unsuitable for pond stocking of postlarvae over the 'winter' period. A nursery phase is therefore probably essential to produce PL's late in the breeding season that will generate large juveniles for stocking ponds when pond water temperature rise in spring. Consequently, the current study was designed to investigate the effects of manipulation of stocking density and shelter availability, as well as any possible interactions of the two factors, on

survival, growth rate and productivity of newly settled postlarvae of an Australian *M. spinipes* experimental culture stocks.

5.3. Materials and Methods:

5.3.1 Source of Postlarvae

Broodstock for the trial were originally collected from the Flinders River system and its tributaries, in the Gulf of Carpentaria region (latitude 17°52.522; longitude 140°46.837) of north Queensland, Australia and had been confirmed belonging to Lineage II by the Queensland University of Technology (de Bruyn *et al*., 2004b). Broodstock used for production of PL's for the current experiment were first generation of captive bred prawns collected from an experimental growout pond held at the Freshwater Fisheries and Aquaculture Centre, Queensland Department of Primary Industries and Fisheries (QDPI&F), located at Walkamin, north Queensland. The pond was stocked previously with PL's produced at the James Cook University (JCU) hatchery from broodstock prawns collected from the wild. Baited traps were sued to capture large prawns that weighed from 60 to 80 g that were transported back to the Marine and Aquaculture Research Facility Unit (MARFU), JCU, Townsville where they were held in two recirculating 3,000 L tanks. Individuals were fed daily *ad libitum* on shrimp, mussel, squid and a formulated feed (36 % crude protein, 6 % crude fat and 3 % crude fibre) designed for black tiger prawn *Penaeus monodon* (Ridley Aqua-feed, Australia). Appearance of berried females was monitored closely and when identified they were transferred to 300 L hatching tanks filled with 5 – 8 ‰ brackish water for incubation.

Larval culture was carried out in a conical 400 L tank with newly hatched larvae stocked after hatching at a salinity of 12.0 ± 1.5 ‰. Larvae were fed newly hatched *Artemia* nauplii (INVE Inc, Thailand) daily with live microalgae *Nannochloropsis* sp. added regularly to create 'green water' culture conditions with algal concentrations maintained at >1,000,000cells/ ml, particularly during the first half of the larval culture cycle (Lober and Zeng, 2009). When > 90 % larvae in the culture tank were estimated to have reached PL stage, salinity in the culture tank was slowly reduced to 4 ‰ over the course of 6 hours. Newly settled PL's were then harvested and transferred to outdoor oval 1,000 L tanks where they were held for one week to acclimate to freshwater before being used for the current experiment. PL's were fed daily on a commercial prawn starter # 1 crumble feed (43 % crude protein, 6 % crude fat and 3 % crude fibre) designed for black tiger prawn (Ridley's Aquafeed, Australia) over the trial period.

5.3.2 Experimental design and setup

To assess the effect of stocking density and shelter availability on survival and growth rate of PL's and final productivity of *M. spinipes*, six treatments of PL's stocked at 3 initial densities of 500, 1000 and 2000 PL m^{-3} with or without shelter, respectively, were setup. All treatments were in quadruplicate where each replicate consisted of a designated number of PL (40, 80 and 160 PL for a density of 500, 1000 and 2000 PL m^{-3} respectively) stocked in a 100 L square glass aquaria (58cm L x 58cm W x 30cm H) filled with 80 L dechlorinated freshwater to create designated stocking densities. A total of 24 aquaria were used. All aquaria were connected to a freshwater recirculation system and a 1000 L sump with a water recirculation rate set at approximately 200 L per hour. The bottom and sides of the aquaria were lined with black plastic on the outside to prevent potential visual interference among juveniles in different aquaria and the top of each aquarium was covered with a removable mesh screen to restrict escapes. For each replicate of

'with shelter' treatments, 14 strips (50 cm W x 15 cm L) of extruded, heavy duty and rigid black plastic mesh (20mm mesh size) were added as shelters. Shelters were tied radially to terracotta square weights (3 or 4 pieces of strips tied to one weight) to ensure they remained on the bottoms of tanks. Shelters were placed vertically to the bottoms of the tanks to allow juveniles to cling to. As a result, the surface area provided by these shelters increased the total internal tank surface area by approximately 1.01 times. Aeration was provided to each aquarium and submerged heaters were placed in the sump to maintain water temperature at 28 ± 2°C across the experimental period. Photoperiod was set at 14.5L: 9.5D.

PL's used for the experiment were weighed (12 ± 4 mg) at the commencement of the experiment and the experiment lasted for 60 days. PL's were fed initially with a starter # 1 crumble feed (43% crude protein, 6% crude fat and 3% crude fibre) designed for black tiger prawn *P. monodon* (Ridley's Aquafeed, Australia) but halfway through the experiment, a starter # 2 feed (43% crude protein, 6% crude fat and 3% crude fibre) was offered as young prawns had increased in size. A feeding ration at 10% of prawn body weight was adopted at the beginning of the experiment but the ration was gradually reduced to only 5% of body weight towards the end of the experiment. Uneaten feed and faeces was siphoned out each morning. Water quality parameters, including DO, pH, NH_3/ NH_4^+, NO_2^-, NO_3^-, were checked weekly and a 100 % water exchange was carried out fortnightly.

5.3.3 Data collection and analysis

Over the 60 day experiment duration, growth data were collected every 10 days via random sampling of 20 juveniles from each replicate (i.e. total 80 per treatment) for to measure individual

length and wet weight. Total length was measured using Vernier digital callipers (0.01 mm). Individual wet weight was measured with a digital scale (Ohaus Adventurer Pro; 0.001 g). Sampled juveniles were gently blotted dry with tissue paper before being placed individually in a small plastic container filled with water that had been tared beforehand. After measurement, all prawns were returned promptly to the aquarium from which they had been sampled. At the end of the experiment, number of surviving juvenile prawns was counted for each replicate to obtain the final survival rate. Individual prawns were then measured for wet weight and total length. They were then placed in a 60 °C oven for 24 h prior to dry after which dry weight was estimated using a Sartorius TE2145 (0.1 mg) balance.

Final survival was arc sin transformed while biomass, wet and dry weight data were log transformed prior to statistical analyses and homogeneity of variance was tested using Levene's test. All data were subjected to two-way ANOVA analysis and if a significant difference was detected, Tukey's post hoc test was employed to identify which treatments were significantly differentfrom other treatmetns. The significance level was set at 0.05 (Sokal and Rohlf, 1995). Data were presented as untransformed values for ease of interpretation. All data analysis was carried out using SPSS Version 16.0.

5.4. Results

5.4.1 Water quality

Table 11 summarises all water quality parameters monitored over the course of the 60 day culture period. Overall, levels of ammonia (NH_3/ NH_4^+ mg/ L), nitrite (NO_2^- mg/ L) and nitrate (NO_3^-

mg/ L), pH, dissolved oxygen (mg/ L) were all within the acceptable range for juveniles of *M. rosenbergii* (Tidwell *et al*., 2004; Tidwell *et al*., 2003).

5.4.2 Effects of stocking density and shelter on survival

At the end of the 60 days trial, there was a clear trend of juvenile survival rate decreasing as stocking density increased (Tab. 12). Since no significant interaction was detected ($P>0.05$) between stocking density and shelter availability on survival rate, survival rate results from the two same density treatments (i.e. with and without shelter) were pooled for further analysis (Tab. 12). The highest mean survival rate (84.8 %) was recorded in the low density treatment of 500 PL m^{-3}, followed by the intermediate density of 1000 PL m^{-3} (74.4 %) while the high density treatment of 2000 PL m^{-3} showed the lowest survival (57.4 %). Statistical analysis confirmed that survival rate in the high stocking density (2000 PL m^{-3}) treatment was significantly lower than that of the intermediate density (1000 PL m^{-3}) and the latter had a significantly lower survival rate compared with the low density treatment (500 PL m^{-3}) ($P<0.05$) (Tab. 12).

For, with shelter and no shelter treatments, average survival rate of with shelter treatments (77 %) was higher than that of the without shelter (67 %) treatments, however the difference was not statistically significant ($P>0.05$) (Tab. 3).

5.4.3 Effects of stocking density and shelter on growth rate

Changes in total length and wet weight of individual juveniles at 10 day intervals are presented in Table 13. Overall, differences in wet weights in different treatments began to appear after 10 days of culture with wet weights of 0.04 ± 0.001 g for all treatments and the differences kept increasing after each subsequent 10 day sampling period. Despite increasing weights over the culture period,

no significant differences ($P>0.05$) in juvenile wet weights were detected between the 10 day intervals.

At the end of the 60 day culture cycle, we observed a trend for increasing mean individual wet weight of juveniles at higher initial stocking density (Tab. 12). Since no significant interaction ($P>0.05$) was detected between stocking density and shelter availability on growth rate, data were pooled for each density with and without shelter treatments. On average, the heaviest juveniles (144.24 mg) were produced in the high stocking density treatment (2000 PL m^{-3}) while the 1000 and 500 PL m^{-3} treatments produced lighter juveniles (124.61 and 117.99 mg, respectively). Average wet weights of juveniles in the 500, 1000 and 2000 PL m^{-3} treatments were significantly ($P<0.05$) different from each other with both 500 and 1000 PL m^{-3} treatments producing significantly ($P<0.05$) lighter juveniles than those sampled from the 2000 PL m^{-3} treatment (Tab.12). When treatments with and without shelter availability were compared, treatments with shelters provided the mean wet weights of juveniles (134.28 mg) were significantly heavier than that of treatments where no shelter (123.61 mg) was provided (Tab. 12). T

Average total length of juveniles from the 2000 PL m^{-3} (26.36 mm) were significantly ($P<0.05$) different from both the 500 (25.29 mm) and 1000 PL m^{-3} (25.64 mm) stocking density treatments. The higher stocking density of 2000 PL m^{-3} produced longer juveniles than was evident in the two lower densities. Average total length of juveniles in the 500 and 1000 PL m^{-3} treatments however were not significantly ($P>0.05$) different from each other. Results of juvenile total lengths in the shelter treatment (26.07 mm) were significantly different ($P<0.05$) from those without shelter treatment (25.36 mm). Juveniles in the with shelter treatment were significantly ($P<0.05$) longer than those from the without shelter treatment.

Individual dry weight of juveniles were significantly ($P<0.05$) different in different stocking density treatments as well as between treatments with and without shelter ($P<0.05$) (Tab. 12). The intermediate stocking density treatment 1000 PL m^{-3} produced the heaviest mean dry weight (35.05 mg), while the 2000 PL m^{-3} produced an intermediate dry weight (34.18 mg) and the 500 PL m^{-3} produced the lowest mean dry weight (32.93 mg). Mean dry weights from the different stocking density treatments were all significantly ($P<0.05$) different from each other. For treatments with and without shelter, the without shelter treatment (34.34 mg) produced a heavier mean juvenile dry weight when match with shelter (33.76 mg) and both were significantly different from other treatments ($P<0.05$) (Tab. 12).

5.4.4 Effects of stocking density and shelter on final biomass and the increase in biomass

Regardless if shelter was available, final biomass of all surviving juveniles after 60 days of culture were higher in the high stocking density treatment (Tab. 12). No significant interaction was evident between stocking density and shelter availability, allowing final biomass among densities to be compared separately. The high density 2000 PL m^{-3} treatment produced the highest total biomass (13.45 g), was followed by the 1000 PL m^{-3} treatment (8.22 g). The low density 500 PL m^{-3} treatment generated the lowest mean biomass (4.69 g), that was about half of that of the 1000 PL m^{-3} treatment and one third that of the 2000 PL m^{-3} treatment. Statistical analysis showed that final biomass production was significantly different at all three stocking density treatments ($P<0.05$) (Tab. 12).

When treatments with and without shelter addition were compared, the mean biomass produced in treatments with shelter were significantly higher (11.76 g vs 8.79 g) ($P<0.05$) than without shelter

(Tab. 12). Similarly, increase in biomass over the culture period showed a pattern parallel to that with increase in stocking density. The high density treatment (2000 PL m^{-3}) produced the highest increase in biomass (8.87 g), followed by the 1000 PL m^{-3} treatment (5.29 g) while the lowest increase (2.95 g) was found in the 500 PL m^{-3} treatment, and accounted for approximately only one third that of 2000 PL m^{-3} treatment. Biomass increases in the three stocking density treatments were significantly different from each other ($P<0.05$ (Tab.12). Comparison of with or without shelter treatment, showed a higher increase in biomass (6.93 g) with shelter and this was significantly different ($P<0.05$) from the without shelter treatment) (Tab. 12).

Table 11. Range of water quality parameters recorded during the 60 day culture of newly settled postlarvae of Australian *Macrobrachium spinipes* stocked at different initial densities, and with or without added shelters, respectively.

Water quality parameter	Range
Dissolved oxygen (mg/L)	5.52 – 7.00
pH	6.4 – 8.2
Ammonia (NH_3/ NH_4^+) (mg/L)	0 – 0.25
Nitrite (NO_2^-) (mg/L)	0
Nitrate (NO_3^-) (mg/L)	0.1 – 5.0

Table 12. Main effect means for newly settled postlarvae of Australian *Macrobrachium spinipes* cultured for 60 days at three initial stocking densities and with or without added shelters. Mean (± SE) of eight replicate aquaria for each stocking density and, twelve replicate aquaria for with and without shelter treatments.

Main effects	Survival (%)	Total length (mm)	Wet weight (mg ind^{-1})	Dry weight (mg ind^{-1})	Final total biomass (g)	Increase in Biomass (g)
Stocking Density						
500 PL m^{-3}	84.75 ± 0.98 ^{a}	25.29 ± 0.19 ^{a}	117.99 ± 0.003 ^{a}	32.93 ± 0.002 ^{a}	4.69 ± 0.16 ^{a}	2.95 ± 0.15 ^{a}
1000 PL m^{-3}	74.38 ± 2.6 ^{b}	25.64 ± 0.18 ^{a}	124.61 ± 0.003 ^{b}	35.05 ± 0.002 ^{b}	8.22 ± 0.46 ^{b}	5.29 ± 0.41 ^{b}
2000 PL m^{-3}	57.38 ± 8.22 ^{c}	26.36 ± 0.29 ^{b}	144.24 ± 0.01 ^{c}	34.18 ± 0.002 ^{c}	13.45 ± 1.39 ^{c}	8.87 ± 1.28 ^{c}
Shelter						
With	77 ± 11.2 ^{a}	26.07 ± 0.2 ^{a}	134.28 ± 0.01 ^{a}	33.76 ± 0.002 ^{a}	11.76 ± 1.84 ^{a}	6.93 ± 1.14 ^{a}
Without	67 ± 7.41 ^{a}	25.36 ± 0.17 ^{b}	123.61 ± 0.003 ^{b}	34.34 ± 0.002 ^{b}	8.79 ± 1.12 ^{b}	4.48 ± 0.54 ^{b}

Different superscripts within a same column indicate significant differences ($P<0.05$).

Table 13: Increases in wet weight (mg ind^{-1}; mean ± SE) of newly settled postlarvae of Australian *Macrobrachium spinipes* cultured under different initial stocking densities, and with or without added shelters, respectively, over 60 days.

	0 days	10 days	20 days	30 days	40 days	50 days	60 days
Stocking Density							
500 PL m^{-3}	0.01± 0.001 ^{a}	0.04 ± 0.001 ^{a}	0.06 ± 0.001 ^{a}	0.07 ± 0.002 ^{a}	0.08 ± 0.002 ^{a}	0.1 ± 0.003 ^{a}	0.1 ± 0.003 ^{a}
1000 PL m^{-3}	0.01± 0.001 ^{a}	0.04 ± 0.001 ^{a}	0.06 ± 0.001 ^{a}	0.08 ± 0.002 ^{a}	0.09 ± 0.003 ^{a}	0.1 ± 0.003 ^{a}	0.1 ± 0.003 ^{a}
2000 PL m^{-3}	0.01± 0.001 ^{a}	0.04 ± 0.001 ^{a}	0.06 ± 0.002 ^{a}	0.07 ± 0.002 ^{a}	0.1 ± 0.004 ^{a}	0.11 ± 0.003 ^{a}	0.1 ± 0.01 ^{a}
Shelter							
With	0.01± 0.001 ^{a}	0.04 ± 0.002 ^{a}	0.06 ± 0.001 ^{a}	0.08 ± 0.002 ^{a}	0.1 ± 0.001 ^{a}	0.1 ± 0.001 ^{a}	0.1 ± 0.001 ^{a}
Without	0.01± 0.001 ^{a}	0.04 ± 0.001 ^{a}	0.06 ± 0.001 ^{a}	0.07 ± 0.001 ^{a}	0.09 ± 0.01 ^{a}	0.1 ± 0.003 ^{a}	0.1 ± 0.002 ^{a}

Different superscripts within a same column indicate significant differences ($P<0.05$)

5.5. Discussion

The results of the study showed that for the nursery culture phase of *M. spinipes*, higher stocking densities resulted in lower survival rates of juveniles. In particular, at an initial stocking density of 2000 PL m^{-3}, survival was significantly lower than at a stocking density of 500 PL m^{-3}. This pattern is expected and can be explained by the fact that at high stocking density, crowded culture conditions increase encounter rates and intensify competition for food and space among juvenile prawns, leading to heightened aggressive behaviour and higher incidents of cannibalism rates (Siddiqui *et al*., 1997; Marques *et al*., 2000; Coyle *et al*., 2003; Tidwell *et al*., 2003;).

Given the aggressive and cannibalistic nature of *M. spinipes*, as has been described for *M. rosenbergii*, with addition of shelters, juvenile prawns have the advantage of additional surface area for perching (Sandifer *et al*., 1983) and a place of refuge from attack by other individuals, especially during, and soon after moulting, when individuals are soft shelled and more vulnerable (Ra'anan *et al*., 1984). With an increase in stocking density, any beneficial effects of shelter addition are likely to become more evident under intensified competition for food and space because individuals are likely to encounter more conspecifics in a densely populated culture environment. Of interest however, interestingly, in the present study, was that even though addition of shelters produced higher survival rates than the same density treatments without shelters, , no significant interaction was detected between stocking density and shelter addition. In parallel, analysis of effects of shelter addition on survival rate separately also showed no significant effects. This however most likely results from high within treatment variation evident at the highest density masking any potential impact of shelter addition (Tab.12).

In fact, the analyses indicated no significant interaction between stocking density and shelter addition for any of the measured variables. This differs from the report by Ra'anan *et al*. (1984) on *M. rosenbergii* growout who reported that shelter addition effects increased at higher densities. In contrast, for a juvenile *M. rosenbergii* pond growout trial, Tidwell *et al*. (1999) did not find a significant interaction between stocking density and substrate availability, but their study did show similar results, on focus here for *M. spinipes,* that at high stocking densities, survival was reduced, but that survival rate was enhanced with availability of added substrate. Such different results obtained from various studies can probably be explained by different density ranges and culture conditions employed as well as life stage and size of animals used in the experiments. In the present study, small newly settled postlarvae were used and because of their relative small size, the density range tested may not have reached any potential threshold that could produce a significant interaction between stocking density and shelter addition. Furthermore, unlike in a growout situation, experimental animals used in the present study were newly settled postlarvae, where individual size variation was minimal (0.01 ± 0.001 mg), this likely helped to reduce incidents of cannibalism.

The quantity, structure and orientation of the shelters could also affect the outcomes. In the current study, tank underwater surface was increased by only 1.01 times with addition of the shelters, so perhaps adding more shelter area could further enhance survival rates, particularly at higher stocking densities (Tidwell *et al*., 2000). Orientation of the shelters in the tanks was vertical in current study, which was adopted mainly for tank maintenance convenience during siphoning, feeding and monitoring. Vertical vs horizontal orientation of shelters did not however affect survival and average weight rates for ungraded *M. rosenbergii* juveniles after 60 days culture by Tidwell *et al*. (2002). While physical characteristics of the substrate used was also not compared

here, a study of this factor on Malaysian *M. rosenbergii* (Tidwell and Coyle, 2008) showed no effect. Hence, potentially cheaper materials can be used as shelters to save on costs. Clearly, further studies on effects of quantity, structure and orientation of shelters on performance of Australian *M. spinipes* during nursery culture will be required.

Intense competition for space and food under high stocking densities is normally expected to impact negatively on growth rate of juvenile prawns. On average, however significantly heavier juvenile wet weights were produced in the high stocking density (2000 PL m^{-3}) compared with that of the low density (500 PL m^{-3}) for *M. spinipes* here. This result most likely can be explained by the significantly higher mortality of juveniles stocked at the high density compared with the low density (57.4% vs. 84.8%) treatments. It is likely that prawns that did not survive in the high density treatment were weaker and smaller, and so easily cannibalised by larger juveniles while in the low density treatment, such prawns had a better chance to survive. As a result, at the end of the experiment the average wet weight of surviving juveniles was higher in the high density treatment than in the low density treatment but there were significantly fewer of them.

Addition of shelters also significantly improved mean wet weight of juveniles when compared to treatments without shelters. Similar results were also obtained by Tidwell and Coyle (2008) after pond growout trials of *M. rosenbergii*. It is likely that addition of shelters into the culture environment served to reduce competition and aggressive encounters between young prawns with more available surface area in 3-dimensions that allowed more efficient utilisation of the water column (Ra'anan, *et al*. 1984; Tidwell *et al*., 2005). Addition of shelters could also potentially provide substrate for periphyton growth, which may provide some extra source of food and

nutrition for young prawns that may enhance their growth rate (Tidwell *et al*., 1997; Tidwell *et al*., 1999).

As the sum of the effects of initial stocking density, mortality and growth rates over the culture period, the total biomass at harvest represents the final relative productivity of the nursery culture phase. The final total biomass was highest in the high stocking density of 2000 PL m^{-3} and was almost 3 times higher than that of the low density treatment of 500 PL m^{-3}. The medium density also produced a significantly higher final total biomass than that evident in the low density treatment. Hence, despite the low stocking rate of 500 PL m^{-3} showing the best survival rate of the three stocking densities tested, in practical aquaculture, it may not be commercially viable due to a low final biomass yield as well as low average weight of juvenile prawns produced. In parallel, addition of shelters also significantly improved total biomass production, which has also been reported for pond growout trials of *M. rosenbergii* (Tidwell *et al.,* 1999; Tidwell and Coyle 2008).

Increases in wet weight of sampled juveniles did not show any significant differences at the three stocking densities tested or between treatments with shelter or without shelter. Note however, significant within treatment variation was evident at the 2000 PL m^{-3} stocking density.

In summary, the present results show overall that the higher stocking densities tested (1000 and 2000 PL m^{-3}) produced better results for nursery culture of Australian *M. spinipes* than did low density and is therefore recommended for commercial nursery production. These stocking rates were also recommended by New (2002) for primary nursery culture of *M. rosenbergii* and another freshwater prawn species *M. dacqueti*. The actual density to be adopted may depend however on facilities available and the situation of each nursery. For instance, if the main objective of a

nursery is to achieve high productivity per culture unit, then higher density within the range reported here could be adopted. Alternatively, if survival rate of the postlarvae is the main concern, then a lower density would be appropriate. To obtain the best performance results, shelters should also be provided in tanks since this is likely to significantly improve final production as well as the individual weights of young prawns produced.

Chapter 6. General Conclusions and Discussion

The current project has been the most comprehensive study to evaluate the factors that will allow optimisation of larval production and early stage growout of the freshwater prawn *Macrobrachium spinipes* (Schenkel, 1902) in Australia. In general, the results are encouraging and provide a foundation for viable production of PL's from simple hatchery systems. The study demonstrated that adult *M. spinipes* tolerance to low temperatures (as low as 12° C) under ambient conditions and highlighted the possibility for culture of the species under low to moderate temperatures. The very high survival of brooders confined in cages over a long period suggests that broodstock can be overwintered successfully under natural conditions. While we were not able to induce spawning of *M. spinipes* outside of the natural breeding season by temperature and photoperiod manipulation, trialling eyestalk ablation to induce out of season spawning may prove worthwhile. Moreover, we will also need to develop better biological information about the target species *M. spinipes* because it would appear to possess some major biological differences from its congener the well studied and widely commercially farmed, *M. rosenbergii*. Undoubtedly these datacan help to define the basic requirements for effective hatchery and growout of the species and promote the development of an industry in North Queensland for the species. In particular, basic biological parameters need to be characterised for *M. spinipes* broodstock management that include fecundity estimates, egg hatchability estimate and larval quality assessment. Current practices employed for *M. rosenbergii* include selection of large size females as breeders under the assumption that they possess higher fecundity. It is not known however, if larval quality is affected by this practice and so female age needs to be further investigated. Evaluation of the requirements for broodstock nutrition will also help identify the basic dietary requirements (i.e. for protein, carbohydrate, triglycerides, cholesterol, phospholipids, vitamin and minerals) in *M.*

spinipes with the aim of improving the quality of larvae. Optimal water quality parameters and environmental tolerance levels (including for ammonia, nitrites, nitrate, dissolved oxygen and salinity) for holding broodstock and berried females should also be assessed to maximise broodstock survival and health. The culture potential of the three remaining lineages of *M. spinipes* within Australia (I, III and IV) also need to be evaluated to see if they have characteristics that would be useful in culture e.g. for high growth rate, cold tolerance (Lutz, 2003), reduced larval culture duration, disease resistance and less size variation during growout to improve productivity (New, 2005). Currently any assumptions about this are only based on what is known about *M. rosenbergii* populations.

Larval culture applying 'greenwater' culture methods with addition of microalgae that exceed a threshold concentration (2.5×10^5 cells/ ml) was shown to significantly improve survival, development and growth of *M. spinipes*. In contrast a 'clearwater' method showed lower larval survival and poorer growth results. Addition of microalgae concentrations as high as 10×10^6 cells/ ml did not result in any negative impacts suggesting higher values could be trialed. Enrichment of *Artemia* however produced only had minimal effects on larval survival rate but did have significant effects on larval growth rate and fatty acid content. Effects of enrichment however diminished effects with increasing microalgal density and hence was suggested to have more application to improve larval survival under 'clearwater' culture. While the underlying mechanism(s) behind significantly improved larval performance with addition of high concentrations of microalgae were not investigated here but one possible explanation is that a dense microalgal environment may provide less stressful underwater lighting conditions and provide a backdrop for more efficient feeding by the larvae while improving water quality via algal secretion of bioactive chemicals or some synergistic effects. Current commercial larval

culture of *M. rosenbergii* mostly employs the 'clearwater' method due to ease of management because the use of a'greenwater' method requires that microalgae cultures need to be maintained. This requires additional resources including appropriate infrastructure and is labour intensive. As the current study suggested that the 'greenwater' culture method only produced significantly improved results for *M. spinipes* when algae addition had reached a threshold level, investigations to examine if similar thresholds exist for *M. rosenbergii* may prove beneficial to its aquaculture worldwide. While water quality parameters used in this study for the larval experiments were extrapolated from *M. rosenbergii* and proved generally appropriate, the optimal ranges of water quality parameters for larval culture of *M. spinipes* should be investigated further and established to enhance hatchery performance. Larval feed for freshwater prawn culture has been dependent on live *Artemia* nauplii and problems related to supply and quality of *Artemia* cysts in crustacean aquaculture open up opportunities to explore alternative larval feeds such as formulated diets.

Nursery culture of *M. spinipes* stocked at different densities with newly settled postlarvae for 60 days showed higher survival when medium and low densities (500 and 1000 PL m^{-3}) were employed. The use of a high stocking rate (2000 PL m^{-3}) in nursery culture however produced heavier and larger-sized juveniles, while addition of shelter also significantly affected final mean wet weight and final biomass at harvest. Depending on the production goals, low densities produce better survival rates while high densities produce greater production. Developing nursery culture for *M. spinipes* would be beneficial to obtain healthy juveniles for growout that could take full advantage of the warmer months over spring/ summer for growout. As size variation was evident during nursery culture, size grading of juveniles during nursery culture could minimize size variability and potentially significantly increase mean harvest size and total production. This should be investigated for *M. spinipes*. Other culture technologies can also be explored including

all-male or monosex culture and selective harvesting of large individuals could be trialled to determine effects on relative growout productivity of *M. spinipes*.

The nutritional requirements for nursery and juvenile growout culture of *M. spinipes* need to be characterised and optimised for feed development that is economical under Australian conditions. Feed comprises a major cost in prawn farming (up to 60%), as substitution of fishmeal with plant proteins and carbohydrates including copra cake, soybean meal and wheat flour have been found to be good sources of nutrients and are digested well by adult *M. rosenbergii* freshwater prawns (Law *et al*., 1990). Research on development of a similar plant-based grow-out diet utilising agro-byproducts available locally in northern Australia should be investigated as well as the use of inducing natural productivity of ponds to optimise natural food availability while lowering the overall production costs.

In summary, the results of this study have demonstrated that the egg to juvenile stage of hatchery production of *M. spinipes* is possible and potentially offers similar opportunities to *M. rosenbergii*.

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Appendix 1

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Effect of microalgae concentration on larval survival, development and growth of an Australian strain of giant freshwater prawn *Macrobrachium rosenbergii*

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ABSTRACT

The present study investigated the effects of microalgae *Nannochloropsis* sp. addition and concentration on larval survival, development and growth of an Australian strain of *M. rosenbergii* (lineage II). Newly hatched larvae were reared to postlarval (PL) stage under the condition of no algae addition ('clear water') and four *Nannochloropsis* concentrations of 2.5, 6.25, 12.5 and 25×10^5 cells/ml. All treatments were in quadruplicate and each replicate had 30 larvae stocked in a 5L vessel. Larvae were fed 3 *Artemia*/ml throughout with 100% water exchange daily. The results showed that larval survival to PL at the two higher algae concentrations of 12.5 and 25×10^5 cells/ml (70.8 and 63.3%, respectively) were significantly higher ($P<0.05$) than those of lower algae concentrations of 2.5 and 6.25×10^5 cells/ml and the 'clear water' treatments (26.7, 35.0 and 30.0%, respectively). Meanwhile, the fastest mean development to PL (30.6 days) registered at the highest algal density was 14 days shorter than that of the 'clear water' treatment (44.3 days). Larval development at the two higher algal densities were significantly shorter than that of the 'clear water' treatment and larval development of the highest algal density was further significantly faster than those of the two lower algal densities (40.1 and 40.0 days) ($P<0.05$). The mean dry weights of newly settled PL of the two high algal density treatments were also significantly heavier ($P<0.05$) than those of the lowest algal density and the 'clear water' treatments. The results have shown that the addition of *Nannochloropsis* sp. at appropriate levels substantially improved performance of larval culture of the Australian strain of *M. rosenbergii*, suggesting that the Australian native strain has a promising potential for aquacultural development.

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, has long been part of the diet of the peoples of Asia and the Pacific who greatly value its flavour. The extensive farming of *M. rosenbergii* employing various traditional methods has a long history in Southeast Asia where it occurs naturally (Ling, 1969). The development of modern culture techniques for the species in the 1960's and 70's has led to the introduction of the species to many countries and farming of the species is now wide spread throughout the world wherever appropriate climate and freshwater impoundments exist (New, 2002, 2005).

The natural distribution of *M. rosenbergii* extends from Pakistan across to Southeast Asia, south to Papua New Guinea, northern Australia (De Bruyn et al., 2004a) and in some Indian and Pacific Ocean islands (Mather and De Bruyn, 2003). In Australia, the giant freshwater prawn is widely distributed throughout the tropical northern regions and endemic to the west of the Great Dividing Range (Short, 2004). Several attempts in the past to grow local *M. rosenbergii* commercially in the country failed, reporting various problems, including low hatchery survival, excessive cannibalism, lack of technical expertise and infrastructure to consistently produce postlarvae (Cantrelle, 1988) as well as diseases that have affected commercial operations (Bergin, 1986; Owens and Evans, 1989). As a consequence of such failed attempts and strict government regulations that prohibit introduction of foreign species/strains to Australia for aquaculture, commercial freshwater prawn farming is currently non-existent in the country.

The worldwide upsurge in *Macrobrachium* culture in the past decade has prompted research interest again in Australia, particularly on the problematical hatchery phase. These new efforts were further encouraged by recent reports, which through the analyses of 16sRNA, divided natural populations of *M. rosenbergii* into 'eastern' and 'western' forms, demarcated by Huxley's Line, a biogeographic barrier running between Borneo and Sumatra extending north into the Philippines (De Bruyn et al., 2004a). Based on this finding, *M. rosenbergii* in Australia belong to the 'eastern' form, differing from the widely cultured 'western' form of mainly Malaysian origin (De Bruyn et al., 2004a). Further analysis of mitochondrial DNA revealed that within Australia, wild stocks of *M. rosenbergii* can be categorized into four genealogically distinguished lineages, i.e. Western Australia

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(lineage I), Gulf of Carpentaria/Northern Territory (lineage II), Irian Jaya (lineage III) and Papua New Guinea/North east Cape York (lineage IV) (De Bruyn et al., 2004b).

In line with these new findings, in the attempts to assess the aquaculture potential of various Australian native strains of *M. rosenbergii*, wild broodstock of Lineage II were sourced from the Flinders River system, North Queensland and larval culture trials were carried out based on techniques derived from the 'western form' (New, 2002). As both the 'clear water' and 'green water' methods have been used for larval culture of the 'western form' of *M. rosenbergii* and no clear verdict has been made as to which was superior (New, 2002), both methods were trialled during several larval culture runs for the Australian strain of *M. rosenbergii* in identical tanks to compare their relative merits. Interestingly, contrary to the current trend of commercial hatcheries overseas opting for the 'clear water' method (New, 2002), results from our trials suggested that the 'green water' method consistently produced significantly better results for the Australian strain of *M. rosenbergii*. Anecdotal observations further suggested that larval performance was linked to the density of microalgae added. Hence, the present study was designed to quantitatively assess the effects of algae addition on larval survival, development and growth of the Australian strain of *M. rosenbergii*.

2. Materials and methods

2.1. Source of broodstock and larvae

Broodstock prawns were collected from the Flinders River system and its tributaries, near the Gulf of Carpentaria (latitude 17°52.522; longitude 140°46.837), North Queensland, Australia. The prawns were transported overnight in 200 L black plastic drums with aeration to the Marine and Aquaculture Research Facility Unit (MARFU), James Cook University (JCU), Townsville, Queensland. Upon arrival at MARFU, broodstock prawns were held in recirculating 2500 L tanks at a female to male ration of 4–5:1. The prawns were fed daily in excess on shrimp, mussel, squid and formulated feed (36% crude protein; 6% crude fat and 3% fibre) designed for black tiger prawn *Penaeus monodon* (Ridley Aqua-feed, Australia). Samples of these wild prawns were sent to Queensland University of Technology (QUT), Brisbane for identification and were confirmed as Lineage II from rivers flowing into the Gulf of Carpentaria (De Bruyn et al., 2004b).

The development of ovaries and spawning of the female prawns were monitored closely. Berried females were transferred to 5–8‰ brackish water for incubation and embryonic development monitored to predict the date of hatching. On the day of hatching, broodstock females were removed from the hatching tank after larvae had hatched and salinity in the tank was increased to 12‰. Newly hatched larvae were held for 1 h at 12‰ before being collected and transferred to experimental containers for the experiment.

2.2. Experimental design and setup

Five larval culture treatments were setup with concentrations of green microalgae *Nannochloropsis* sp. set at five levels of 0, 2.5, 6.25, 12.5 and 25×10^5 cells/ ml. All treatments were in quadruplicate where each replicate consisted of a 5 L round clear plastic container (diameter=215 mm; depth=177 mm) stocked with 30 randomly selected newly hatched larvae in 12±1‰. Replicates of various treatments were organised in a random block design inside water baths set at 30.0±1.5 °C. Each container was covered with a clear plastic lid to prevent larvae jumping out during the late larval stages and gentle aeration was provided to each container via a fine-tipped glass pipette inserted through the lid. Photoperiod was set at 14.5 L: 9.5D throughout the experiment and larvae were cultured from the day of hatching (day 0) until they either reached postlarval stage (PL) or death in all replicates.

Throughout the experiment, larvae were fed a ration of 3 *Artemia*/ml with 100% water exchange carried out daily. Water was exchanged in the morning where any mortality was recorded and the surviving larvae of each replicate were transferred to an identical container with freshly prepared food (3 *Artemia*/ml and designated concentration of *Nannochloropsis*) and 12‰ water. The water salinity was pre-adjusted by diluting natural seawater of 33–36‰ with dechlorinated tap water using a refractometer (Shibuya Salinometer S-10). Water quality parameters, including ammonia, nitrite, nitrate, pH and DO, were measured weekly. Over the period of the experiment, the ammonia, nitrite and nitrate ranged from 0–0.3, 0–0.1 and 0–10 mg/L, respectively, while pH fluctuated between 7.4 to 7.9 and DO between 5.7 to 6.3 mg/L.

Microalgae *Nannochloropsis* sp. was mass cultured in 3000 L tanks at JCU's algal culture facility using a commercially available fertiliser (AQUASOL, Yates Ltd, New South Wales, Australia). The *Nannochloropsis* cultures were generally re-inoculated every 7–10 days. During the experiment, a stock solution was prepared daily by selecting a *Nannochloropsis* sp. culture at its exponential phase of growth and diluted to 12‰ by mixing with dechlorinated freshwater in a 20 L container with strong aeration. Samples were then taken from the stock solution and the algal density counted using a hemocytometer under a high power microscope. The *Nannochloropsis* concentration of the stock solution was estimated by averaging the concentrations of five 1 ml samples. The volume of stock solution required to prepare a desired concentration of microalgae for each treatment was calculated using the equation:

$$C_1V_1 = C_2V_2$$

where C_1 was the designated algal concentration for a particular treatment and V_1 was the total volume required for daily water exchange of the treatment; C_2 was the algal density of the stock solution and V_2 was the volume of the stock solution required to prepare the designated algal concentration for the particular treatment. The required volume of the stock solution was subsequently measured and diluted with 12‰ brackish water to prepare the total volumes required for daily water exchange. Meanwhile, *Artemia* cysts (INVE Inc, Thailand) were hatched daily in 18‰ and newly hatched nauplii were harvested early morning on the following day and their density counted before being fed to the larvae directly without enrichment.

Every 3 days, 16 larvae (4 from each replicate) were randomly sampled from each treatment and their developmental stage identified under a microscope according to Uno and Kwon (1969). The larvae were placed in a small pool of water during the stage identification and returned to the original culture promptly after staging. Such a process has been shown previously not to lead to larval mortality when operated properly. Once postlarvae were found in a replicate during the daily check, they were removed from the cultures and euthanized by quick freezing. The larvae were then measured for their carapace length (mm) using a microscope equipped with a camera (Leica). They were subsequently dried individually in a 60 °C oven for 24 h before being weighed for dry weight using a Cahn C-33 microbalance (0.001 mg).

2.3. Data analysis

Based on results of larval staging, larval stage index (LSI) was calculated according to Manzi et al. (1977) and Mallasen and Valenti (2006):

$$\mathrm{LSI} = (\sum S_i \times n_i)/N$$

Where S_i=larval stage (i=1–11; representing each larval stage); n_i= number of larvae in stage S_i; N=total number of larvae examined. The survival, mean development time from hatching to PL, mean carapace

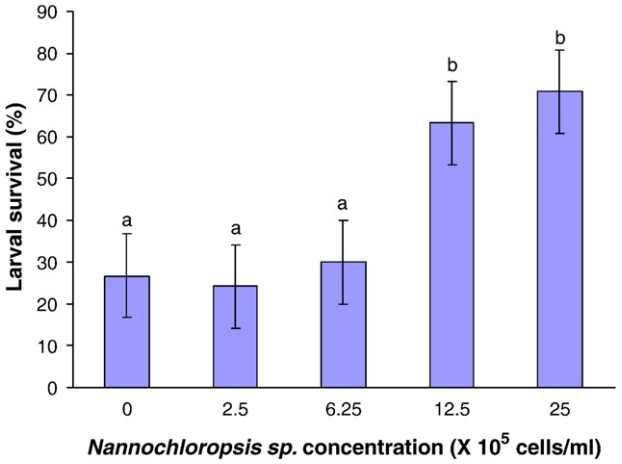

Fig. 1. Cumulative larval survival from hatching to postlarvae of an Australian strain giant freshwater prawn *Macrobrachium rosenbergii* (Lineage II), cultured under different concentrations of *Nannochloropsis* sp. Data are presented as mean ± SD (n=4). Different superscripted letters indicate significant differences (P<0.05).

length of PL were analysed using one-way ANOVA while mean dry weights of newly settled PL were log transformed prior to analysis with one-way ANOVA. Tukey's test was employed to detect significant differences among treatments at the 0.05 significance level (Sokal and Rohlf, 1995). All data analysis was carried out using statistic's package, SPSS Version 16.0.

3. Results

3.1. Larval survival

Fig. 1 shows percentage survival of newly hatched larvae to PL stage of the different treatments. The effects of *Nannochloropsis* addition on larval survival were evident: the highest survival (70.8%) was obtained for the highest microalgae concentration of 25×10^5 cells/ml, followed by the second highest microalgae concentration treatment of 12.5×10^5 cells/ml (63.3%). Substantially lower survival was recorded from the 'clear water' treatment (30.0%) and two lower microalgae density treatments of 2.5 and 6.25×10^5 cells/ml (26.7% and 35.0%, respectively). Statistical analyses showed that larval survival of the two high algal treatments were significantly better than that of the other 3 treatments of lower or no algal addition (P<0.05), however, no significant differences was detected between the two highest algal treatments and among other three treatments (Fig. 1).

Interestingly, the plot of mean daily larval survival of various treatments showed that for the first 5–6 days, larval survival were all very high and not much different from each other. The major departing in larval survival occurred mainly over the period of day 7 to 20, during which larval survival of the two high algal treatments remained >80% while those of the low algal and 'clear water' treatments dropped substantially (Fig. 2).

Table 1
Mean development time to postlarval stage (PL), carapace length and dry weight of newly settled postlarvae cultured at different microalgae (*Nannochloropsis* sp.) concentrations

Nannochloropsis sp. concentration (cells/ml)	Mean development time from hatching to PL (days)*	Carapace length of newly settled PL (mm)*	Dry weight of newly settled PL (mg)*
0	44.3 ± 4.0^{c}	3.03 ± 0.44^{a}	0.676 ± 0.177^{b}
2.5×10^5	40.0 ± 5.2^{bc}	3.03 ± 0.39^{a}	0.676 ± 0.124^{b}
6.25×10^5	40.1 ± 3.1^{bc}	2.92 ± 0.31^{a}	0.704 ± 0.156^{ab}
12.5×10^5	33.2 ± 3.5^{ab}	3.06 ± 0.20^{a}	0.852 ± 0.241^{a}
25×10^5	30.6 ± 2.1^{a}	3.05 ± 0.25^{a}	0.852 ± 0.204^{a}

*Different superscripted letters of a same column indicate significant differences (P<0.05).
Data are presented as mean ± S.D. (n=4).

3.2. Larval development

Larval development, as measured by mean time required to develop to PL, showed a clear trend of improvement with increased algae addition (Table 1). The fastest development registered at the highest algal density (25×10^5 cells/ml) was about 14 days shorter than that of 'clear water' treatment. Statistics showed that larval development at the highest algal concentration was significantly faster than those of two lower algal concentrations (2.5 and 6.25×10^5 cells/ml) and the 'clear water' treatment while the larval development of the second highest algal treatment (12.5×10^5 cells/ml) was also significantly shorter than that of the 'clear water' treatment (P<0.05) (Table 1). Furthermore, larval development appeared to be more synchronised at the highest algae concentration of 25×10^5 cells/ml, as indicated by a substantially reduced standard deviation (Table 1).

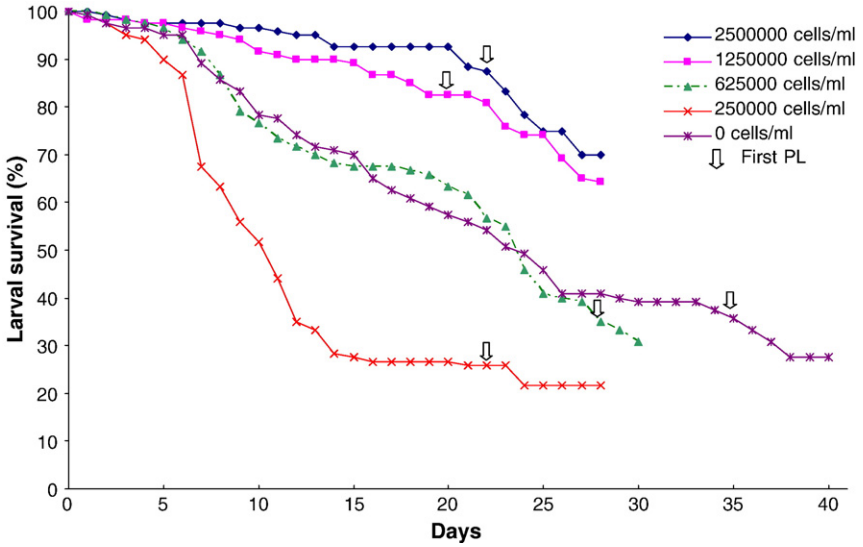

Fig. 2. Daily percentage survival of larvae of an Australian strain giant freshwater prawn *Macrobrachium rosenbergii* (Lineage II), cultured under different concentrations of *Nannochloropsis* sp. Note that postlarvae survival is not included and the survival line of a treatment terminated on the day that all larvae in the treatment became PL or were dead.

Table 2
Larval stage index (LSI) for larvae cultured at different microglae (*Nannochloropsis* sp.) concentrations

Nannochloropsis concentration (cells/ml)	Day 2	Day 5	Day 8	Day 11	Day 14	Day 17	Day 20	Day 23**	Day 26	Day 29	Day32	Day 35	Day 38
0	2.8±0.1^{a}	4.1±0.5^{b}	4.9±0.4^{b}	5.9±0.5^{c}	6.4±0.9^{b}	7.1±0.9^{b}	7.7±0.8^{c}	7.9±1.0^{a}	7.9±0.7^{b}	8.7±0.7	8.9±0.4	8.8±0.6	9.5±0.7
2.5×10^5	2.5±0.7^{a}	4.3±0.6^{b}	5.1±0.5^{ab}	6.2±0.3^{c}	7.5±0.3^{ab}	7.8±1.0^{ab}	8.3±0.5^{bc}	8.9±0.4	9.3±0.2	9.8±0.7	9.9±0.5	10.1±0.3	–
6.25×10^5	2.5±0.7^{a}	4.8±0.1^{ab}	5.3±0.4^{ab}	6.8±0.4^{bc}	7.4±0.3^{ab}	7.4±0.3^{ab}	7.7±0.5^{c}	8.2±0.7^{a}	8.9±0.4^{a}	8.7±0.4	9.1±0.3	9.5±0.5	–
12.5×10^5	2.8±0.2^{a}	4.8±0.4^{ab}	5.7±0.1^{ab}	7.3±0.4^{ab}	7.9±0.5^{a}	7.9±0.5^{ab}	9.0±0.5^{ab}	9.2±0.6	9.6±0.3	10.4±0.6	9.9±0.8	–	–
25×10^5	3.0±0.0^{a}	5.1±0.1^{a}	6.1±0.7^{a}	7.9±0.5^{a}	8.3±0.4^{a}	8.3±0.4^{a}	10.1±0.1^{a}	10.2±0.4	10.4±0.4	10.6±0.3	10.5±0.3	–	–

*Different superscripted letters of a same column indicate significant differences ($P<0.05$).
**After first appearance of PL, treatments were excluded from the statistical analysis and the data presented after the first PL are for reference only as all PL were removed from culture and not included for the calculation of LSI.

The larval stage index (LSI) calculated based on larval samples taken throughout the culture showed a general trend of higher LSI value with increased algal concentration. Significant differences of LSI occurred as early as day 5, when the LSI of the highest algal concentration was significantly higher than those of the lowest algal concentration and the 'clear water' treatments (Table 2). The first PL appeared on day 20 and 22 for higher algal treatments of 12.5 and 25×10^5 cells/ml, respectively, it was on day 28 and 35 that the first PL was found in the lower algae concentration of 6.25×10^5 cells/ml and the 'clear water' treatment, respectively (Fig. 2). At 2.5×10^5 cells/ml, the first and second PL's were observed relatively early on day 22 and 23, respectively, however, unlike in other treatments where subsequent PL's were found on the following days, the 3rd PL of the treatment appeared only 7 days later, on day 30. The LSI data showed that the first metamorphosis to PL generally occurred at LSI ≤9 for all treatments except that it was 10 for the highest algal density treatment, further suggesting a more synchronised development under the culture condition.

3.3. Postlarval dry weight and carapace length

The dry weight of newly settled PL generally increased with elevated microalgae concentration (Table 1). Dry weights of PL from the two high algal concentrations were the same at 0.852 mg, about 20.6% heavier than the lowest dry weight (0.676 mg) recorded for the lowest algal density and the 'clear water' treatments. The dry weight of PL from the 6.25×10^5 cells/ml treatment was intermediate at 0.704 mg. Statistics showed that dry weights of the two high algal concentration treatments were significantly higher ($P<0.05$) than those of the lowest algal density and the 'clear water' treatments. In contrast, no significant differences in mean carapace lengths of PL was found among all treatments (Table 1).

4. Discussion

The current study clearly demonstrated that the addition of *Nannochloropsis* sp. at appropriate levels led to significantly improved larval survival, development and growth of the Australian strain of *M. rosenbergii*. Larval survival to PL was significantly higher at the two higher algal levels of 12.5 and 25×10^5 cells/ml, suggesting that between the algal levels of 12.5×10^5 cells/ml and 6.25×10^5 cells/ml, lies a critical threshold. At the highest algal concentration of 25×10^5 cells/ml, development to PL was the shortest and significantly faster than all other treatments except that of the second highest algal density of 12.5×10^5 cells/ml. This was also reflected in the LSI values, with generally higher LSI recorded for the higher algal concentrations. Significant differences in LSI were registered as early as on day 5, suggesting that the beneficial effects of algae started rather early.

Furthermore, the improved growth at the two higher microalgae levels was manifested by significantly heavier PL dry weights. It is worth noting that the carapace length of PL was not significantly different among treatments, suggesting that carapace length is not a good indicator for larval growth. Based on the present results, it is recommended that for larval culture of the Lineage II of *M. rosenbergii*, microalgae *Nannochloropsis* sp. should be added at a level ≥12.5×10^5 cells/ml.

There are several possible explanations for the observed beneficial effects of adding high levels of *Nannochloropsis* to larval culture of the Australia strain of *M. rosenbergii*. Firstly, the addition of *Nannochloropsis* may provide better nutrition to the larvae. Manzi et al. (1977) reported that algal cells were found in the gut of larvae of the 'western form' of *M. rosenbergii*, however, they were unsure whether those cells were actively consumed or accidentally ingested. These authors further pointed out that it appeared there was no evidence of any overt assimilation of the algal cells and direct nutrition to the larvae (Cohen et al., 1976; Joseph, 1977). Judging by the carnivorous nature of *M. rosenbergii* larvae, the nutritional benefits of direct ingestion of algal cells by larvae is probably limited.

Alternatively, larvae may benefit nutritionally through ingesting *Artemia* grazing on abundance of algae. *Artemia* are the major live feed for *M. rosenbergii* larvae, however, *Artemia* is known to lack some essential nutrients, particularly the highly unsaturated fatty acids (HUFA), such as decosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), that are important for the survival and development of fish and crustacean larvae (Léger et al., 1986). The HUFA content of *Artemia* therefore often determines the food value of *Artemia*. Despite lack of DHA, *Nannochloropsis* sp. is known to be rich in EPA (Volkman et al., 1989), which may contribute to better nutritional content and quality of *Artemia* as food for the freshwater prawn larvae. However, to what extent such contribution was is unknown as *Nannochloropsis* sp. is not considered a particularly suitable diet for *Artemia* nauplii due to toughness and the indigestibility of its cell wall (Dhont and Lavens, 1996).

Secondly, the green microalgae *Nannochloropsis* may provide a culture condition that is less stressful to *M. rosenbergii* larvae and at the same time, may aid their feeding. There is ample evidence that underwater lighting conditions and tank colour could play an important role in foraging success of fish larvae (Ostrowski, 1989; Tamazouzt et al., 2002), far less attention has been paid to crustacean larvae, probably because of the fact they are capable of feeding in the darkness, therefore, implying a feeding mechanism by either random encounter or chemosensory detection (Rabbani and Zeng, 2005). However, such ability does not preclude the possibility that in the presence of light, crustacean larvae may utilise visual cues to enhance their foraging and feeding efficiency (Rabbani and Zeng, 2005). Such a hypothesis is supported by two recent reports that showed tank colour significantly impacted larval survival of the 'western form' of *M. rosenbergii* (Yasharian et al., 2005) and the mud crab *Scylla serrata* (Rabbani and Zeng, 2005). Interestingly, Yasharian et al. (2005) reported that for the 'western form' of *M. rosenbergii*, when the 'clear water' method was used, larvae cultured in red and green tanks had the best and significantly higher survival than those reared in white and blue tanks. This agrees with the finding of AQUACOP (1977) who reported best larval survival from dark green tanks. It was suggested that as visual ability and active predatory behaviour increases with development, the ability to effectively pinpoint the food items become

increasingly important; the green background probably provided better contrast or silhouette for the food items, hence improved larval feeding success, survival and growth (Yasharian et al., 2005). The addition of green algae *Nannochloropsis* may have a similar effect on larval feeding as the green coloured tanks. This is also in agreement with the present results that significant improvement in larval performance mainly occurred when high concentrations of *Nannochloropsis* were added. In a separate study, Lin and Omori (1993) found that feeding rates of larval *M. rosenbergii* decreased with the increase in lightness of the rearing container. They found that the speed and distance of horizontal movement of the larvae increased by 3 times in the white containers compared to those in black containers. Yasharian et al. (2005) suggested that such excess 'excitation' behaviour may contribute to lower feeding rates and that white background may represent a chronic stressor to the larval prawn. Similarly, the addition of *Nannochloropsis* may serve to reduce the stress and allow larvae to feed more readily and effectively.

Finally, adding microalgae to larval culture may help improve water quality via a reduction in ammonia and other nitrogen wastes in the culture medium (Cohen et al., 1976; New, 2002). However, considering that the water used in the current experiment was totally exchanged daily, a built up of toxic nitrogen compounds seems less likely to be a major limiting factor. However, there is also a possibility that microalgae may secrete unknown bioactive chemicals that inhibit various pathogens or directly benefit the larvae. Obviously, the beneficial effects of adding *Nannochloropsis* to *M. rosenbergii* larval culture could be multiple with added-on or synergistic effects, which is an interesting area that warrants further research. Whatever the underling mechanisms may be, the results of this study clearly show that larvae reared in high microalgae densities performed significantly better for the lineage II Australian strain of *M. rosenbergii*. It strongly suggests that microalgae addition is important to larval culture of this strain of *M. rosenbergii* and there is a threshold level to be reached before such beneficial effects become apparent.

Microalgae have also previously been reported to have a significant role in the success of the larval culture of the 'Western form' of *M. rosenbergii*. The pioneer work of Fujimura (1966) and Fujimura and Okamoto (1972) highlighted the importance of using microalgae and *Artemia* nauplii in larviculture as it increased larval survival and reduced the time to achieve PL. Other researchers also confirmed that unialgal supplements, such as *Isochrysis galbana* and *Tetraselmis suecica*, in combination with *Artemia* nauplii improved larval growth and survival (Wickins, 1972). Manzi et al. (1977) further reported that algal supplements, particularly those of the Chrysophta family (i.e. *Isochrysis galbana, Pseudoisochrysis paradoxam* and *Phaedactylum tricornutum*) significantly increased survival and PL production in both static and recirculating culture systems. Unfortunately, the optimal level of microalgae was not established experimentally by these experiments. For example, algal concentrations were reported to range from 20,000 to 1,200,000 cells/ml with an average of 340,000 cells/ml by Manzi et al. (1977). More recently, New (2002) recommended that *Chlorella* sp. to be added at a range of 750,000 to 1,500,000 cells/ml for green water hatchery culture of *M. rosenbergii* larvae. Overall, these ranges of algal levels used for *M. rosenbergii* larvae culture are comparatively higher than those used for marine shrimp, such as *Penaeus stylirostris* and *P. vannamei*, for which diatom *Chaetoceros gracilis* concentrations between 30,000 and 100,000 cells/ml have been reported to assure good growth and survival of the zoeal larvae (Simon, 1978).

Despite earlier research showing beneficial effects of the 'green water' method for the 'Western form' of *M. rosenbergii* larvae, commercial hatchery operations have reportedly moved away from the 'green water' method in favour of the 'clear water' method (New, 2002). A possible explanation for this trend is probably the perceived extra requirements for facilities, labour and expertise for the 'green water' method as well as its management complexity. Another reason could be that, as demonstrated by this study, if the algal concentration did not reach a required threshold, its beneficial effects is relatively limited, which could well sway a hatchery manager to abandon the practice. There were however indications that the adaptation of 'green water' culture may be more crucial for the 'eastern form' of *M. rosenbergii* as 'clear water' generally produced <30% larval survival for the lineage II larvae in our cultures while substantially higher survival has been reported for the 'western from' of *M. rosenbergii* with 'clear water' culture (AQUACOP, 1983). Whether strain differences exist, a systematic reassessment of the pros and cons of the 'clear' vs 'green water' methods for the hatchery culture of the 'western form' of *M. rosenbergii*, including identifying optimal concentrations, may be worthwhile.

Our results clearly showed that hatchery culture of the Australian strain of *M. rosenbergii* is technically feasible when the 'green water' method is adopted. The establishment of the 'green water' method based on microalgae *Nannochloropsis* sp., a euryhaline, hardy species suitable for large scale outdoor culture, could also prove beneficial. Overall, the larval survival and development of this strain are comparable to those of the 'western form'. For example, using the 'green water' method, Manzi et al. (1977) reported survival ranging from 75 to 82% for the 'western form' of *M. rosenbergii*. First PLs were observed in the current cultures from day 20 to 35 in various treatments, similarly, 18 to 35 days were reported for the 'western form' (Manzi and Maddox, 1977; Manzi et al., 1977; Malecha et al., 1980). The slightly lower survival recorded in the current experiment is most likely due to differences in culture conditions, such as substantially smaller culture vessels used (1 L vs. 60 L) and high stress levels caused by regular handling during daily water exchange and regular larval staging in the current experiment. In fact, in our larval culture trials using large 400–500 L tanks, survival has consistently been higher than 85% with PL production as high as 170 PLs/L culture water. PLs also appeared as early as day 18 with >95% larvae reaching PL by day 24–26. The promising larvae culture results of this Australian strain of *M. rosenbergii* not only show potential for the development of a freshwater prawn aquaculture industry in Australia, but also possible cross breeding programmes with other strains/form for the improvement of productivity of *M. rosenbergii* worldwide (New, 2005).

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