

***Macrobrachium rosenbergii* (de Man 1879): the
antennal gland and the role of pheromones in
mating behaviour**



**UNIVERSITY OF
STIRLING**

A thesis submitted for the degree of Doctor of Philosophy

by

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Declaration

I hereby declare that the work and results presented in this thesis were carried out by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not previously been submitted for any other degree or qualification. All information from other sources has been acknowledged.

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Dedication

To my parent with full love for their continued patience and support whom without them I would not exists. To my family who gave time, patience and support to my son Abdullah and my daughter Rahaf who both showered me with their loves and

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Abstract

The freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879) is an important aquaculture species but one that has the disadvantage of heterogeneous individual growth (HIG) according to different morphotypes. Chemical cues, especially, pheromones, are one of the most important communication types between individual prawns, along with visual and tactile methods. Testing pheromones, whilst restricting other cues, may therefore lead to a better understanding of the influence of these communicatory compounds on the prawn reproductive process. The three principle objectives of this study were therefore: 1) To examine the effect of moult stage and morphotype on pheromone-induced sexual behaviour 2) To examine the role of pheromone / urine concentrations on sexual attraction behaviour 3) To describe the functional morphology of the antennal gland and examine its possible role in pheromone production and release.

Identical bioassay tanks were designed and constructed to study the reproductive behaviour of prawns. Experiments were set up to examine responses to pheromone release by live prawns over 30 minutes and behavioural response observations were made with the aid of a Closed-Circuit Videotape System (CCVS). Results were statistically analysed using a repeated measures general linear model (GLM). Three trials were designed to test the effect of moult stage of both males and females and male morphotypes on sexual attraction behavioural responses. Twelve prawns were used for each trial and each prawn was used five times (1 no-pheromone control and 4 for experimental tests). The first trial studied the effect of female moult stages (pre-, inter and newly-moulted) on sexual attraction behaviour of blue claw (BC) male. Results of

this trial showed that newly-moulted females spent significantly ($p<0.05$) less time approaching the BC male than the pre- and inter-moult females. The second trial studied the effect of male moult stage (pre-, inter and newly-moulted) on sexual attraction to receptive females. Results showed that the time taken by the inter-moult males was ($p<0.05$) less than the pre- and newly-moulted males in approaching the newly-moulted female. The third trial tested the effect of male morphotypes (small male, SM, orange claw, OC and dominant blue claw, BC) on sexual attraction behaviour towards newly-moulted females. Results showed that the BC male was significantly more attractive ($p<0.05$) than other morphotypes to newly-moulted females and that the OC male was the least attractive.

The role of moulting stage for both male and female prawns on reproductive response behaviour was investigated. Because BC males responded significantly faster towards newly-moulted female more than to either pre-or inter-moult females, results of the first trial suggest that BC males are able to use different chemical cues to gather information about a conspecific's gender and can differentiate female's moult stages. Since BC males responded significantly faster towards newly-moulted females more than to either pre-or inter-moult females, this suggests that females at this particular stage released a distinct sexual pheromone or concentration of pheromone that differed from those pheromones released by both pre- and inter-moult females.

In contrast, newly-moulted females prefer the inter-moult BC males which indicate that females have an ability to distinguish the moult status of BC males. Furthermore, it indicates that pheromone characteristics change with the moult status of BC males.

Also, newly-moulted females are most likely to be avoiding the potential costs of mate guarding with soft shell BC males.

Results obtained from the third trial suggested that a newly-moulted female can discriminate male morphotypes (SM, OC and BC) from their pheromone cues. This indicates that male morphotypes release pheromones which differ from each other in some way. Newly-moulted females responded positively to both SM and BC males with different levels of attraction with the greatest attraction to BC males to BC males suggesting that pheromone released from the BC male may carry information relating to dominance status.

Urine is believed to be one of the main carriers of pheromone and is usually released from the antennal gland. Different urine concentrations (0.1, 1.0, 2.0, 3.0, 5.0 and 10 μ l l⁻¹) of collected urine from BC males were used to test the sexual attraction behaviour of receptive newly-moulted females. Also, the attractant capability of fresh urine following exposure to different temperature regimes (cooled at 4°C, frozen at -70°C and heated at 70°C) was tested.

Since newly-moulted female *M. rosenbergii* were attracted to BC male urine, this indicates the existence of sex pheromone in the fresh urine. Also, it was found that the sexual response of females to fresh urine of BC males was directly proportional to urine concentration with faster responses observed with increasing urine concentrations. At the three fresh urine concentrations 0.1 μ l l⁻¹, 1.0 μ l l⁻¹ and 2.0 μ l l⁻¹, statistical analysis

indicated no significant difference ($p>0.05$) between these three concentrations while a significant ($P<0.05$) response was to concentrations more than $3.0 \mu\text{l l}^{-1}$. This may indicate that these three concentrations were not sufficient to elicit attraction behaviour in newly-moulted females. A concentration of $3.0 \mu\text{l l}^{-1}$ of fresh urine is suggested to be a sufficient concentration to elicit a significant sexual attraction under laboratory conditions.

Response of newly-moulted female prawns to the various temperature treatments tested declined in response to nominally increasingly degradative treatments. Also, statistical analysis showed that temperature treatment and concentration added both had a significant effect on the response of females. The greatest degradation of urine attractiveness was found with the 70°C heat treatment. It can be concluded that the pheromone components of prawn urine are friable when exposed to high temperatures.

Using light and transmission electron microscopes, ultrastructural observation of the antennal gland (AG) of *M. rosenbergii* suggests that it has four distinct regions, the coelomosac, the nephridial tubules, the labyrinth and the bladder. Morphological and functional descriptions of each of these regions were compared with those of other aquatic Crustacea.

Keywords: *Macrobrachium rosenbergii*, freshwater prawn, mating behaviour, moult stage, urine concentration, pheromone, antennal gland.

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Chapter 1 General introduction and literature review

1.1 Background

The Malaysian giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879) is considered as one of the largest size freshwater prawn species for aquaculture. This species is widely-distributed through tropical and subtropical zones (see Figure 1) where its range extends from Australia to New Guinea, and to the Indus River delta (Johnson, 1960; George, 1969). Farming of *M. rosenbergii*, however, has been expanded elsewhere even to temperate zones in different parts of Asia, Africa and both Americas (New, 1990).

In the decades of 1950 and 1960, the demand for *M. rosenbergii* grew very quickly as a result method for its culture was developed (Ling, 1969; Uno and Kwon, 1969; Fujimura and Okamoto, 1972). Aquaculture of *M. rosenbergii* has therefore been established for more than 30 years. World aquaculture production has increased more than ten times from the year 1994 to the year 2006 (FAO, 2008).



Figure 1: Geographical distribution of *M. rosenbergii*. Adapted from Holthuis, L.B. 1980. *FAO species catalogue. Vol.1. Shrimps and prawns of the world.*

Macrobrachium is considered to be one of the largest genera of the order Decapoda belonging to the family Palaemonidae. All “freshwater” prawns, which have been used for aquaculture, belong to the genus of *Macrobrachium*. About 200 species have been described in this genus which mainly lives in freshwater (New, 1990; Holthuis, 2000). However, only 24 species are considered to be fully freshwater and one is marine (*M. intermedium*) (Holthuis, 1980) but the majority such as *M. rosenbergii* (Brown, 1991) require brackish water for part or all their developmental cycle.

Table 1. The table below provides the classification for *M. rosenbergii* according to Wickins (1976); Holthuis (1980) and Bowman and Abele (1982).

Table 1: Taxonomy of *M. rosenbergii*.

Phylum	Arthropoda
Superclass	Crustacea (Pennant, 1977)
Class	Malacostraca (Latereille, 1806)
Order	Decapoda (Latereille, 1803)
Suborder	Natantia (Burkenroad, 1963)
Family	Palaemonidae (Rafinesque, 1815)
Genus	<i>Macrobrachium</i> (Bate, 1866)
Species	<i>rosenbergii</i> (de Man, 1879)

1.2 History

Extensive fish farming was practiced in China as early as 4,000 years ago (Hickling, 1962). However, crustacean aquaculture techniques and research are relatively modern. It has been demonstrated that modern aquaculture of *M. rosenbergii* started in the late 1950s but the exact beginning year and the first person that discovered this modern aquaculture for this species is in debate. Dr. Shao-wen Ling began his research in July 1959 in Penang, Malaysia (New, 2000). Two years later, in June 1961, Ling had discovered that brackish water is essential for the larval survival and development, and then he completed the life cycle (Chew, 1990). Funge-Smith (1991), however, reported that John (1957) was the first person to discover the necessity of saline water for the *M. rosenbergii* life cycle when he observed the absence of larvae in freshwater trawls, but that they were present in brackish water. Dr. Ling's success and publication became a landmark and the technology spread rapidly. Following Shao-Wen Ling's discovery of larval-rearing requirements for the freshwater prawn *M. rosenbergii* in Malaysia during 1961, in 1965, Takuji Fujimura along with his research team applied Ling's discovery to commercial mass production in Honolulu, Hawaii, and they then distributed their techniques to some southern states of the USA (Wong and Brock, 1991). Within a decade, there was considerable growth in interest in culturing *M. rosenbergii* in several countries (New, 2000). Later, significant mass production industries for this species were started in Asia (both Thailand and Taiwan) (New, 2000). From both South-East Asia and Hawaii, hatcheries, brood stock and grow-out research and commercial experiences were introduced to many countries where *M. rosenbergii* was not native (New, 2000).

1.3 World production

Both marine shrimp and freshwater prawn species have become very popular and therefore increasingly important as an aquaculture product. The production of cultured shrimp and prawn around the world is increasing to meet the high market demand. Although, marine shrimp are the most commonly cultured species, the culture of freshwater prawn species, especially *M. rosenbergii*, have become a significant and valuable sector of global aquaculture (New, 2005). *M. rosenbergii* now grows on five continents (Africa, North and South America, Asia and Europe); Asia (particularly China) dominates world aquaculture production. The most recent information (see Table 2 and Figures 2-4) which show that the world aquaculture production of *M. rosenbergii* within twelve years increased from 30,633 MT in 1994 to 210,634 MT in 2006 (FAO, 2008) which indicates the high demand and the importance of this species in freshwater aquaculture.

Table 2: World aquaculture production of the most common marine shrimp species and *M. rosenbergii* from the year 1994 to 2006 (FAO, 2008).

Species		Year	1994	1996	1998	2000	2002	2004	2006
<i>Penaeus vannamei</i>	Tonne		120,581	140,348	193,512	145,386	473,449	1,297,935	2,090,935
	US \$1000		723,583	847,847	1,019,747	792,883	2,284,076	4,484,169	7,720,509
<i>Penaeus monodon</i>	Tonne		559,363	536,891	503,005	630,984	631,571	707,872	637425
	US \$1000		3,896,094	3,860,051	3,215,348	4,518,801	3,631,412	3,361,854	3,041,438
<i>Penaeus indicus</i>	Tonne		10,926	8,668	10,993	16,444	25,774	33,085	28,215
	US \$1000		33,512	31,965	51,802	76,582	133,420	181,719	218,423
<i>M. rosenbergii</i>	Tonne		30,633	67,012	90,133	143,620	188,358	217,893	210,634
	US \$1000		173,616	313,633	393,788	516,752	696,517	968,655	1,045,644

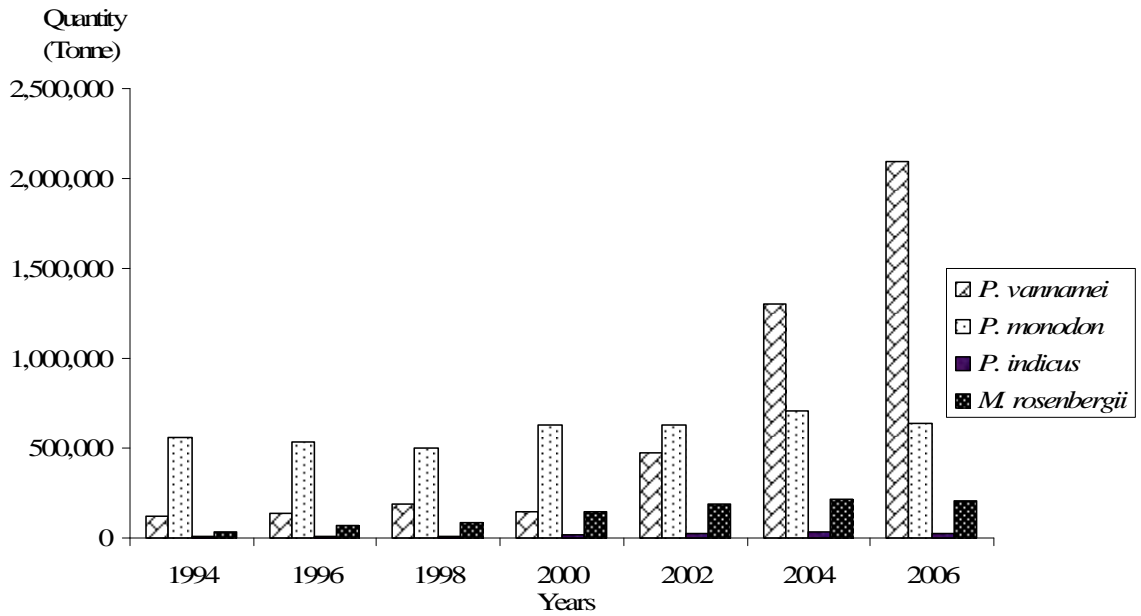


Figure 2: A comparative world aquaculture production quantity (metric tonnes) of the most common marine shrimp with *M. rosenbergii* from the year 1994 to 2006 (FAO, 2008).

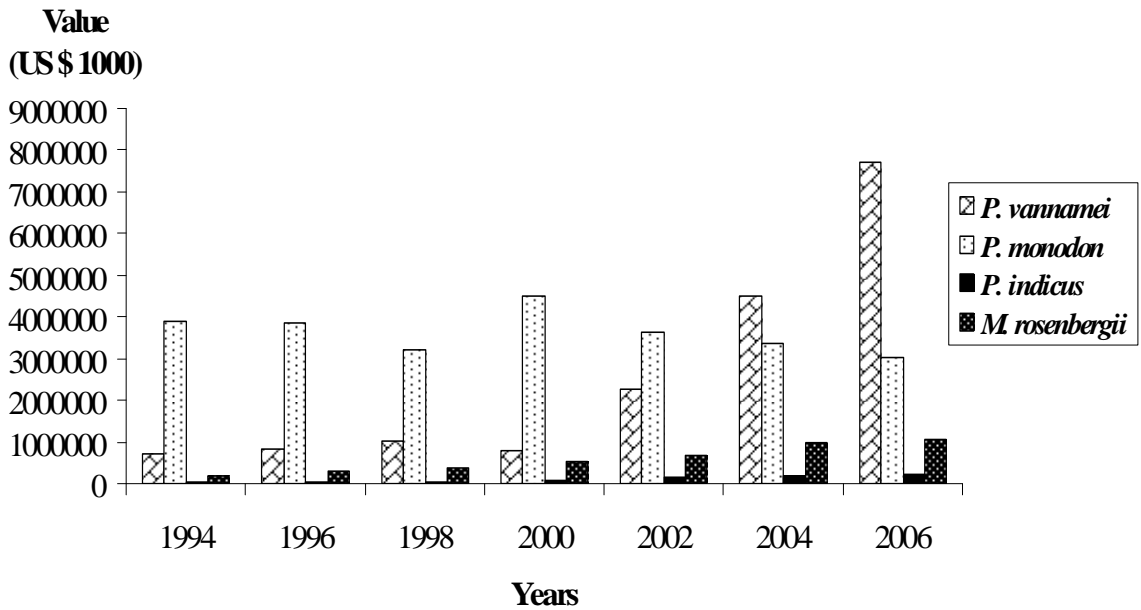


Figure 3: A comparative world aquaculture production value (US \$) of the most common marine shrimp with *M. rosenbergii* from the year 1994 to 2006 (FAO, 2008).

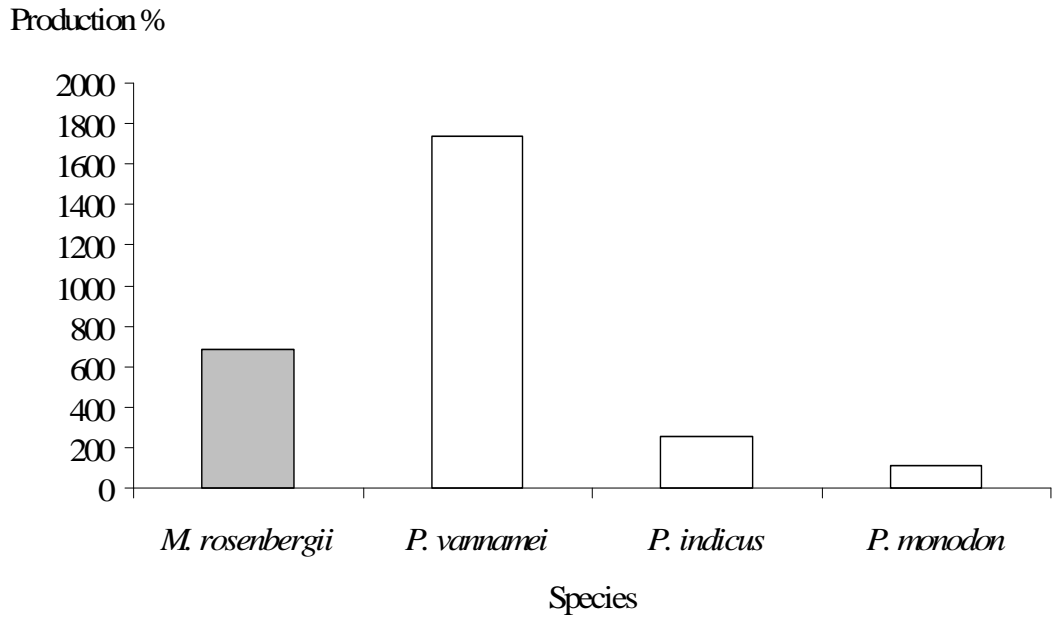


Figure 4: A comparative of world aquaculture production of the most common marine shrimp with *M. rosenbergii* from the year 1994 to 2006 (FAO, 2008).

Table 2 (above) shows that the world aquaculture production of all the four cultured species is increasing, the production rate of *M. rosenbergii* is increasing faster than many marine shrimp species. Also, in the Figure 4 (above) it shows that the world aquaculture production rate from the year 1994 to 2006, *P. vannamei*, *P. monodon*, *P. indicus* and *M. rosenbergii* had increased around 16.3, 0.14, 1.6 and 5.9 times, respectively, (FAO, 2008). This increasing culture of *M. rosenbergii* is probably due to several reasons such as the sustainability for culturing of this species in the inland areas, to their relatively easiness compared to the other marine shrimp species and to fulfil the increasing global market demand.

1.4 Advantages of *M. rosenbergii* culture

M. rosenbergii culture has some real advantages over culture of marine shrimp. Several environmental and social conflicts caused by the negative impacts of poorly-

managed shrimp farming have been reported (Alagarwamy, 1995; Patil and Krishnan, 1997). These include mangrove destruction, salination of agriculture land and consequent impacts on biodiversity, capture fisheries and pollution of coastal waters. Subsequent, to aquaculture development, sea water from shrimp ponds salinised not only the soil but also both the ground and well water which then affected human and agricultural water use. Additionally, in marine shrimp farming there are high risks of viral disease such as the White Spot Syndrome Disease (WSD) which has had severe impact on the success of shrimp farming. Such problems have not been associated with prawn culture.

Freshwater prawn culture in general and that of *M. rosenbergii* in particular became more attractive especially when the shrimp production slowed down in the 1990s. Thus, to some extent, *M. rosenbergii* production compensated for the loss of shrimp production. There are some other advantages of culturing the freshwater prawn. They do not need to be cultured in coastal areas because they spend most of their life cycle in freshwater. In fact, they are often cultured in inland locations, especially the grow-out phase, where they can be closer to large urban markets. In addition, this can be considered as an important source of income for the rural families who live away from the sea coast. The life cycle of this species is uncomplicated and thus it can be easily cultured for the complete life cycle, unlike some other shrimp. *M. rosenbergii* do not appear to be vulnerable to most of the viral diseases that affect marine shrimp species production (Brown, 1991; Tidwell *et al.*, 2003). *M. rosenbergii* has an omnivorous feeding habit which gives them the advantage of wide availability and low costs of feed resources. In addition, *M. rosenbergii* has become a favoured food

source for human consumption in many countries. An additional benefit is that *M. rosenbergii* is a good candidate for commercial polyculture. It has been reared with many species of fish including catfish, tilapia and Chinese carps in the USA (Buck *et al.*, 1981; Behrends *et al.*, 1986), grass, silver carp in Thailand (Tunsutapanich *et al.*, 1982); Chinese carp in Guam (Fitzgerald and Nelson, 1979); bighead and grass carp in Taiwan (Liao and Chao, 1982); channel catfish in Louisiana (Lamon and Avault, 1987) and Mississippi, USA (Heinen *et al.*, 1989); golden shiners in Louisiana, USA (Perry and Traver, 1987); and Nile tilapia and common carp in Saudi Arabia (Siddiqui *et al.*, 1996). Also, *M. rosenbergii* culture has a high potential for integration with other sectoral activities such as agriculture and animal husbandry (e.g. rice field and poultry production) (Zimmerman and New, 2000).

1.5 Aquatic water conditions

Environmental requirements such as water temperature, salinity level, total water hardness, pH level, alkalinity and dissolved oxygen are keys to successful *M. rosenbergii* growth and development. However, temperature is a major factor not only for growth but also to the timing of spawning (Daniels *et al.*, 2000). Many authors have shown that *M. rosenbergii* has quite a narrow range of environmental parameters in which it can thrive. Works of such authors will be demonstrated below at each parameter condition. A review of most common water conditions parameters related to the *M. rosenbergii* will be illustrated as shown below.

1.5.1 Water temperatures

Throughout the literature, it has been reported that there are several rearing temperature ranges for *M. rosenbergii* according to different growth phases. The adult prawns are not only euryhaline but tolerate a wide range of temperature (18°C to 34°C) (New, 1990; Daniels *et al.*, 2000). The optimum temperature range is, however, believed to be from 26°C to 32°C (John 1957; Rao 1967; Sandifer and Smith, 1985; Díaz and Ohno, 1986; New, 1990; Arrignon *et al.*, 1994; Daniels *et al.*, 2000). Daniels *et al.* (1992) suggested that as water temperature decreases, the number of eggs decreases, the time of egg development increases and fungal growth on eggs is promoted. In addition, water temperature plays a role in ovarian maturation, moulting and spawning. The peak spawning temperature of *M. rosenbergii* is from 29°C to 30.5°C (Rao, 1991). A study by Chavez Justo *et al.* (1991) tested the effects of three water temperatures (24°C, 28°C and 32°C) on growth rate and frequency of reproductive moults. They found elevation of water temperatures to 32°C enhanced the growth rate and the female's reproductive moult more than temperatures of 24°C and 28°C. Others, Díaz and Ohno (1986) tested different rearing of temperatures (22°C-34°C) for *M. rosenbergii* and found that the optimal temperature for survival rate to metamorphosis was 28°C. Highest food consumption was found when water temperature was maintained at 32°C. Niu *et al.* (2003) concluded that 33°C was considered to be below or equal to the optimum temperature for growth and food consumption of *M. rosenbergii* post-larvae (PL).

Survival, activity and growth in *M. rosenbergii* are directly affected by surrounding water temperature. A study on the critical minimum and the critical maximum lethal temperatures for post-larvae and juveniles of *M. rosenbergii* (Díaz *et al.*, 1998) found

that they die at 16°C and 42°C. Others, however, found that the mortality increases rapidly at a sustained temperature of less than 19°C and at 33°C and above (Sandifer and Smith, 1985). Low temperatures may slow or stop prawn growth. Akiyama *et al.* (1982) reported that when water temperature is at a range of 18°C–22°C, *M. rosenbergii* growth is inhibited. New (1990) reported that the water temperatures below 14°C or above 35°C are generally lethal for *M. rosenbergii*.

1.5.2 Salinity

Salinity is the presence of dissolved salt content in a body of water. Also, salinity can be defined as a measure of the quantity of dissolved salts in water. *M. rosenbergii* larvae require saline water for their growth and development. The salinity is one of the most crucial factors for the larval rearing phase of *M. rosenbergii* (Daniels *et al.*, 2000). Literature suggested a range of optimal salinity values for *M. rosenbergii* (e.g. 12 - 16‰, Sandifer *et al.* (1977), 8 - 17‰, Sandifer and Smith (1985) and 10 - 15‰, New (1990). Although juvenile and adult stages of this species can live in a relatively wide range of salinity levels, they prefer the freshwater habitat for growth and reproductive activities. In captivity, a salinity of 18‰ could be survived by larval stages (George, 1969). When post-larval stages of *M. rosenbergii* were exposed to high salinities, the mortality began at 25‰ and increased rapidly in salinities of 30‰ and higher (Sandifer *et al.*, 1975). Brooders are usually held in a variety of salinities between fresh to brackish water <15‰ (Daniels *et al.*, 2000). When berried females are kept in brackish water, New (1990) found the hatchability was higher than in freshwater.

1.5.3 Water hardness

The hardness of water is due to the presence of calcium and magnesium minerals that are naturally present in the water. The usual signs of a hard water supply are scaling inside kettles, poor lathering of soaps, and scum. Water hardness is also an important factor affecting survival, growth and reproduction activities of *M. rosenbergii*. Usually, crustaceans require minerals, especially calcium and magnesium, not only for building the exoskeleton (shell) but also for biological processes (Sandifer and Smith, 1985). Cripps and Nakamura (1979), however, tested prawn in 500 mg l⁻¹ of total hardness in water and they observed the moult cycle duration was significantly increased. They believed that precipitation of CaCO₃ in prawns was associated with hardness levels greater than 300 mg l⁻¹. Thus, it is recommended that water hardness level be lower than 300 mg l⁻¹ CaCO₃. Other studies (Cripps and Nakamura, 1979; Vasquez *et al.*, 1989; Arrignon *et al.*, 1994) recommended levels of water hardness to lie within the range of 20 to 200 mg l⁻¹ for optimal *M. rosenbergii* growth. Wickins (1982) suggested that the optimal CaCO₃ concentration in the water should lie between 65 and 200 mg l⁻¹. New and Singholka (1985) recommended that water hardness levels for *M. rosenbergii* should be below 150 mg l⁻¹ and above 40 mg l⁻¹ of CaCO₃ content. However, New and Singholka (1985) preferred that water hardness to be lower than 100 mg l⁻¹. Brown *et al.* (1991) investigated the growth of *M. rosenbergii* juveniles in different ranges of water hardness (9 to 326 mg l⁻¹ CaCO₃) and they found the maximal growth was at a level less than 53 mg l⁻¹ CaCO₃.

1.5.4 pH

The concentration of hydrogen ions is commonly expressed in terms of the pH scale, where it is a measure of the acidity or alkalinity of a solution. Aquatic crustacean

species are vary in their optimal of surrounded water pH scale. Generally, it has been reported that the optimal pH levels for prawns cultured in ponds should lie in the region of 7.0 to 8.5, and should not remain for long periods below 6.5 or above 9.0. The optimum pH of rearing water for *M. rosenbergii* is 7.0 – 8.0 (New, 1990; 1995). Law *et al.* (2002) reported that the optimum pH for *M. rosenbergii* is 7.0. They found that the egg hatchability of *M. rosenbergii* is extremely sensitive to the pH in brackish water. They reported that at 12‰, the highest hatching rate was 92.2% at pH 7.0. When the pH changed to 6.5 or 7.5, the egg hatching rate decreased to 5.0% and 13.3% respectively. However, they observed no hatching at all at pH of 5.0, 8.0, 9.0 and 10.0.

1.5.5 Dissolved oxygen

Prawn requires oxygen for respiration which is acquired from the water by diffusion across the gills. Usually, prawn metabolism adapts to limiting oxygen concentrations by reducing non-obligatory activities such as swimming, processing feed, digesting food and growing (Boyd and Zimmermann, 2000). One of the factors that influence the dissolved oxygen (DO₂) requirements for aquatic crustacean species is body size. Sandifer and Smith (1985) reported that larger individual prawns require more DO₂ than the smaller ones. This means that larger individuals are more vulnerable to low oxygen concentrations. Water temperature plays a significant role in a prawn's respiratory rate, the higher temperature the higher DO₂ consumption. Sharp (1976) (cited in Sandifer and Smith, 1985) found that for a 0.2 g (dry weight) prawn at which the DO₂ concentration is limiting is 2.1 mg l⁻¹ at 23°C, 2.9 mg l⁻¹ at 28°C and 4.7 mg l⁻¹ at 33°C. At lower concentrations, stressful conditions and eventually mortality is likely to occur, however, DO₂ concentrations should always be

maintained above 3 mg l⁻¹ because chronically lower levels of dissolved oxygen throughout the growing season can markedly impact yields. Thus, it is preferable that the DO₂ level in a prawn pond lies between 6–8 mg l⁻¹, but levels as low as 1.0 mg l⁻¹ are not tolerated (Avault, 1987) evidently, when the other culture conditions are at on a best possible levels.

1.6 Morphology

M. rosenbergii was first described in detail by De Man (1879). Morphological description of the main body external parts is shown in Figure 5. *M. rosenbergii* has an elongated body and is circular in cross-section. The prawn's body is usually greenish to brownish grey in colour and consists of two distinct parts: the cephalothorax, which consists of 14 segments (a fusion of the head and thorax), with the first one being vestigial, and the "tail"/abdomen, consisting of 6 segments. The cephalothorax which is covered by a quite hard dorsal shield called the carapace. This carapace ends anterodorsally in a toothed and long rostrum.

In comparison with Penaeidae, as shown in Figure 6 (below) the pleura of the second abdominal pleura of the Palaemonidae, such as *M. rosenbergii* overlap those of the first and third pleura, while in the penaeid shrimp they overlap each other like tiles on a roof (McLaughlin, 1979). In addition, the Palaemonidae have a smooth rounded dorsal abdominal surface of the last abdominal segment (see Figure 7 below) while most Penaeidae have a simple or complex ridge at the dorsal apex of the abdomen (Fincham and Wickins, 1976).

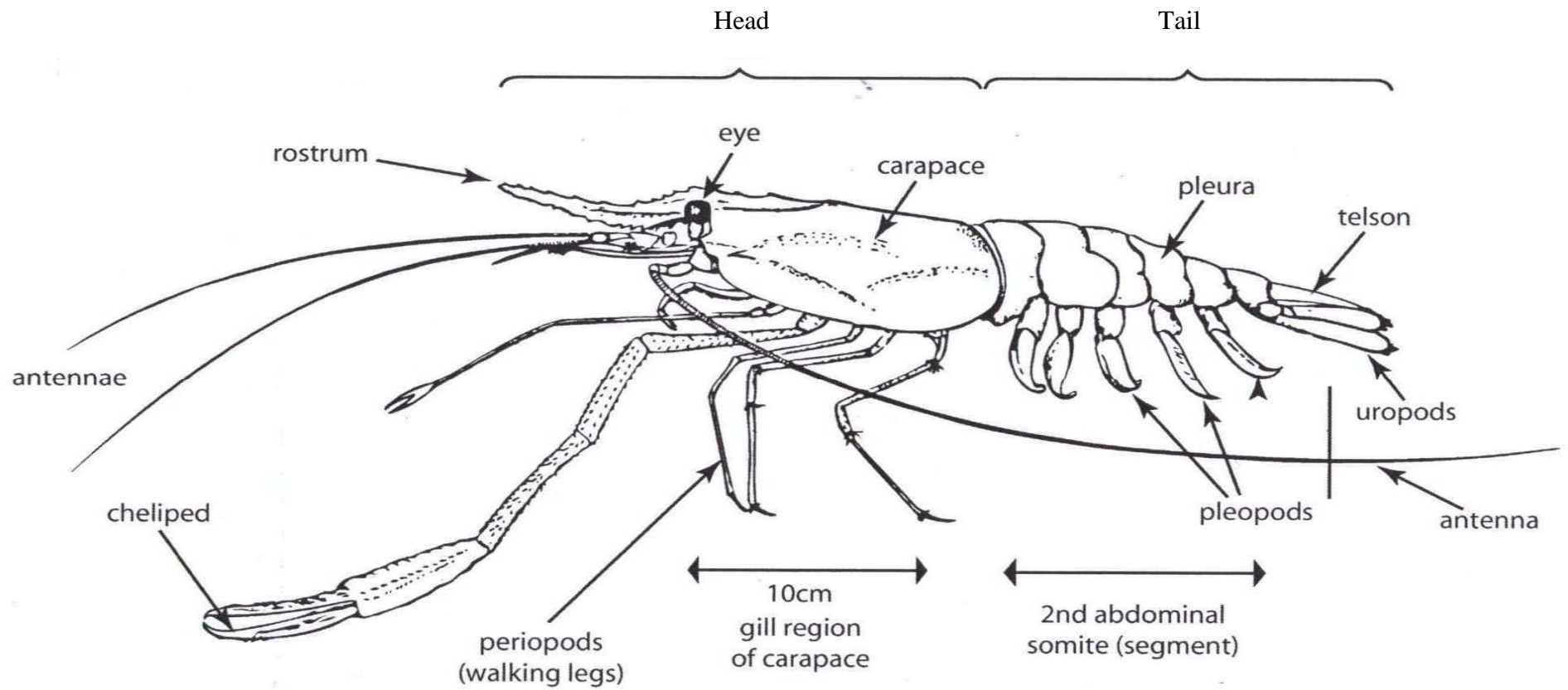
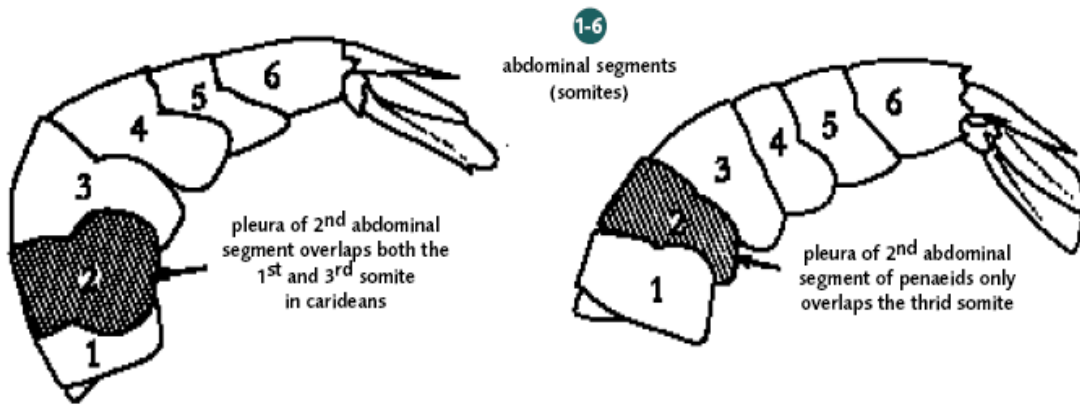


Figure 5: External morphology of freshwater prawn *M. rosenbergii*. Drawing based on Forster and Wickins (1972). Source: New and Singholka, 1985.



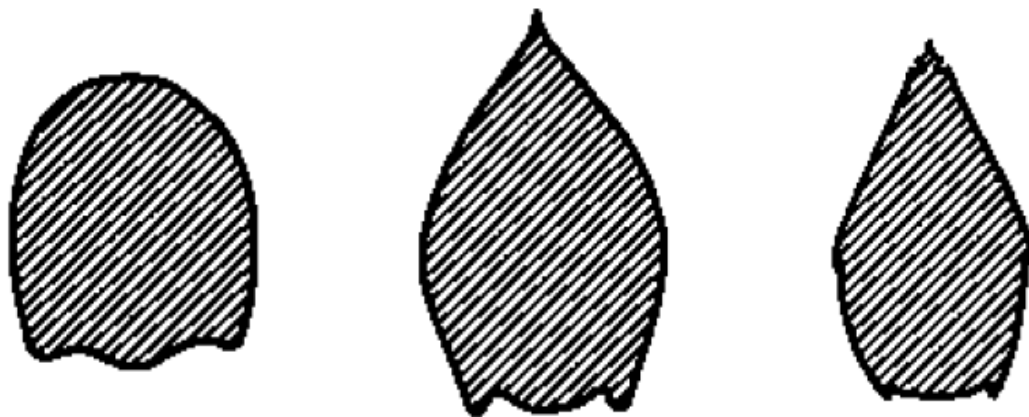
CARIDEA

PENAEIDEA

(includes *Macrobrachium rosenbergii*)

(includes *Penaeus* spp.)

Figure 6: Freshwater (caridean) prawns can also be distinguished from penaeid shrimp by looking at the second pleura on the abdomen (see arrow). Adapted from Emanuela D'Antoni, after Fincham and Wickins (1976), FAO Fisheries and Technical Paper No. 428.



M. rosenbergii

P. latisulcatus

Farfantepenaeus duorarum

Figure 7: The body shape of freshwater prawns (*M. rosenbergii*) is different to that of penaeid shrimp, as these cross sections of the 5th abdominal segments show. Adapted from Emanuela D'Antoni, After Fincham and Wickins (1976), FAO Fisheries and Technical Paper No. 428.

1.7 Morphotypes

Morphotype characteristics, in both genders of *M. rosenbergii* are almost identical from early stages to pre-maturation stage when they tend to be variable, especially males (Nagamine and Knight, 1980; Ra'anan and Cohen, 1985; New, 1990). They can be sexually differentiated when the gonopores in juveniles first appear at 5.9 mm and 7.6 mm Carapace Length (CL) for male and females, respectively. According to Nagamine and Knight (1980), male gonopores are located on the base of the coxae of the fifth pereopods (walking legs) and are covered with flaps. The second pereopods “chelipeds or chelae” are very distinct morphological characteristic between males and females, in which males are larger and thicker than females. Furthermore, on each second pleopod (swimming legs) of the abdomen for both males and females have an appendix interna. However, in addition to the presence of appendix interna, males have an appendix masculina. However, the appendix masculina appears when males reach about 30 mm total length (TL) and is fully developed at size around 70 mm TL (Tombes and Foster, 1979; Nagamine and Knight, 1980). Thus, the presence of the appendix masculina indicates a male while the absence of this particular organ indicates a female. Once reaching sexual maturation, however, males differ from each another and from females in their chelae colour, relative chelae length, chelae thickness and spination (Ra'anan and Cohen, 1985; Kuris *et al.*, 1987; New, 1990) in their reproductive behaviour (Ra'anan and Sagi, 1985) and growth rates (Ra'anan and Cohen, 1985). Differentiation of male morphotypes will be illustrated later in this section.

In contrast, females are, relatively, smaller in size, have smaller heads, shorter and slimmer chelae than males, but they have a uniformity of chelae colour, thickness and

length (Sandifer and Smith, 1985). According to Nagamine and Knight (1980), female *M. rosenbergii* can be distinguished by the presence a pair of gonopores with oval appearance covered with a membrane which are located on the medial surface at the base of the third pereopods (walking legs). Females also have special reproductive setae on the thorax and pleopods (Sandifer and Smith, 1985). Those characteristics can be clearly noticed when the female reaches about 20 mm CL (Nagamine and Knight 1980). They stated that there are two types of reproductive setae that appear on mature females. The first is the ovipositing setae which are located in the posterior margin of the sperm receptacle area and the pleopods. They are mostly permanent, on the coxae of the last three pairs of pereopods where the pleopods guide the eggs during spawning. The second type of setae are the ovigerous which are found only following a pre-spawning moult and their main function appears to affix the fertilized eggs to the pleopods for brooding.

Proportionately, in comparison with females, males have larger heads and the second pereopods are longer, thicker and they have more robust and spinous claws than females (Ling, 1969a; Smith *et al.*, 1980). Males of *M. rosenbergii* can be categorised into three main developmental stages according to their morphological characteristics (Nagamine *et al.*, 1980; Sagi *et al.*, 1986). The first one is small male (SM) in which only the gonopore complexes are present and having a weight range of 1-10g, (~ 6 to 10mm CL) (Nagamine *et al.*, 1980; Sagi *et al.*, 1986). The second one is orange chelae male (OC) in which only the gonopore complexes and appendixes masculina are present and having a weight range of 10-50g (~10 to 28mm CL) which they comprise about 40% of the males (Nagamine *et al.*, 1980; Sagi *et al.*, 1986). Finally, the blue chelae

male (BC) that have gonopore complexes, appendixes masculina, and mature chelipeds present and having the largest weight more than 50g (28mm CL) represent the remaining 10% of the male population (Nagamine *et al.*, 1980; Sagi *et al.*, 1986). Small males and BC actively participate in mating and fertilization, investing little energy in somatic growth, while OC males are characterized by a fast growth rate and reduced reproductive activity (Ra'anana and Sagi, 1985; Sagi *et al.*, 1986).

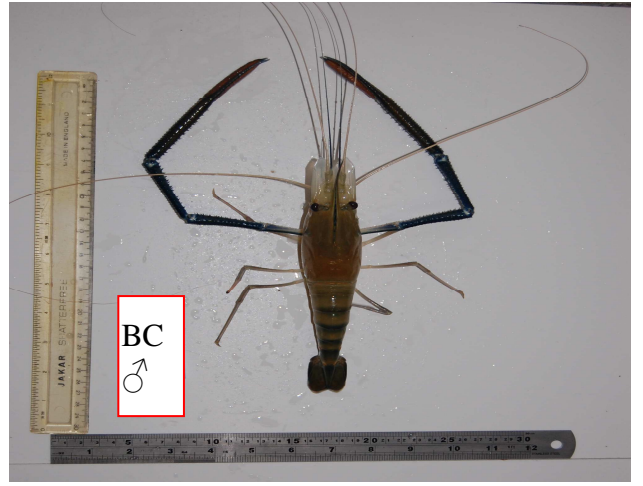
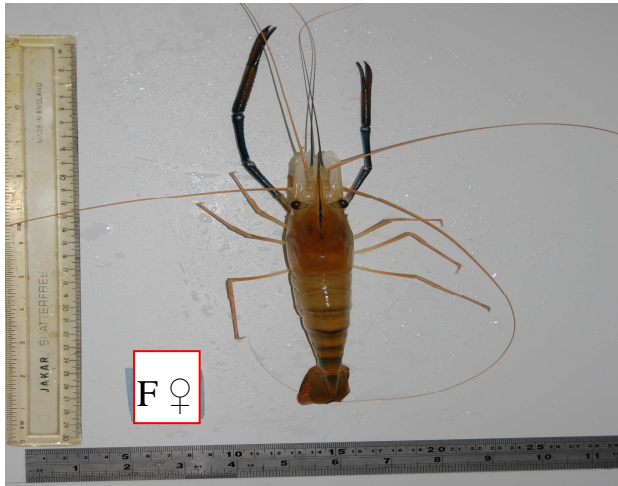
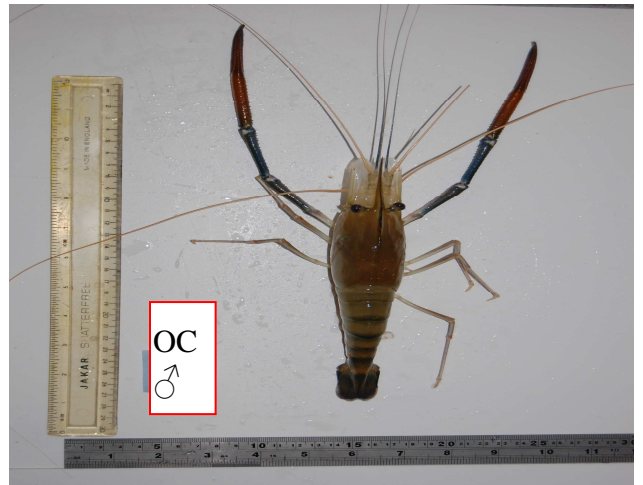
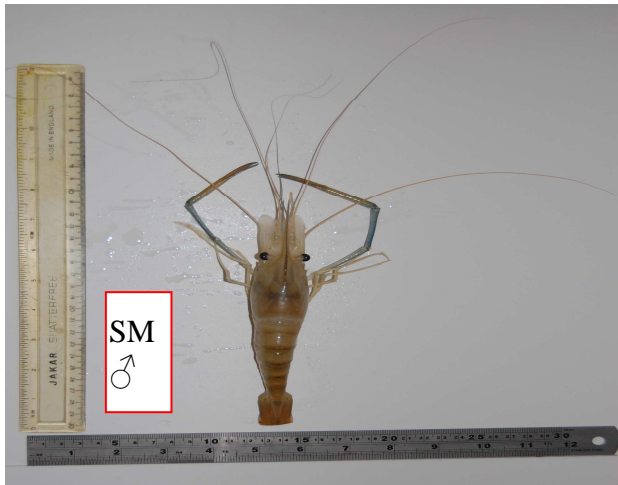


Figure 8: Different morphotypes of *M. rosenbergii*, SM: small male, OC: orange claw male, F: female, and BC: blue claw male.

1.8 Mating

Mating in the giant freshwater prawn *M. rosenbergii* is guided by numerous behavioural and morphological features that promote reproduction between genetically compatible individuals. Mating behaviour in *M. rosenbergii* has been discussed by several authors, including Ling (1969) and Sandifer and Smith (1985). When the surrounding conditions are suitable, mating will occur. However, the behaviour associated with the mating can vary with the social hierarchy. Mating takes place in fresh water where the sexually mature male will flip the female on her back and will align himself above her while holding her in position with his chelae and then depositing a mass of sperm on the female ventral surface between the cephalothorax and abdomen. Mating in *M. rosenbergii* was reported to occur after a pre-mating moult of females (Ling, 1969; Chow *et al.*, 1982). New (1990) described that mating in *M. rosenbergii* takes place between dominant males with a ripe ovaries females that have hard-shell and soft shell, recently moulted female.

1.8.1 Primary mating strategy

M. rosenbergii exhibits different patterns of mating. Each of the three sexually mature male morphotypes has its own mating pattern. Whenever a female becomes receptive to fertilisation, large BC males are able to court actively and protect females prior to and during mating (Ra'anana and Sagi, 1985; Sagi *et al.*, 1988). BC males spend most of the time resting and grooming themselves, otherwise, they are displaying agonistic behaviour against all other approaching individuals or actively courting and protecting the receptive female (Karplus *et al.*, 2000). Ra'anana and Sagi (1985) found the BC is

significantly higher in reproductive success and better in guarding receptive females than the other male morphotypes (SM and OC).

The OC are rapidly growing compared to the SM and BC males and are considered an intermediate phase from the SM to the BC morphotypes (Sagi *et al.*, 1988). Also, it has been reported that the OC males are sub-dominant and were never observed courting and protecting a receptive female (Ra'anana and Sagi, 1985). However, Ra'anana and Sagi (1985) found occasional fertilisations by this morphotype (OC) have had occurred.

Small males, are not territorial, sub-dominant by the BC males and highly mobile consistent with their small size (Peebles, 1980; Sagi *et al.*, 1988). Also, SM, have relatively large reproductive systems (Sagi and Ra'anana, 1988), and their testes contains relatively large amount of mature sperm and are actively engaged in spermatogenesis (Sagi *et al.*, 1988). On several occasions when only SM males were present with females, a successful fertilisation was observed (Ra'anana and Sagi, 1985). It was observed that the sub-dominant SM *M. rosenbergii* employs a sneak copulation strategy (Telecky, 1984). The SM use the sneak mating tactic to avoid direct competition with the larger morphotypes males (BC and OC), which take advantage of their small size and agility to sneak toward the female and quickly transfer spermatophores (Ra'anana and Sagi, 1985). This alternative mating strategy relies on numerous attempts but with relatively little reproductive success (Ra'anana and Sagi, 1985).

1.8.2 Alternative mating strategy

Achieving a high dominant status may require costly resource investments such that individuals who are at a competitive disadvantage may improve their reproductive success by avoiding direct competition and adopting an alternative tactic with relatively lower costs (Dominey, 1984). In the case of polygamous species, however, the reproductive success of males depends on male-male competition and aggression; the suppressed individual males sometimes adopt an alternative mating strategy. Austad (1984) defined the alternative mating behaviour “as any discontinuous variation in an aspect of reproductive behaviour among one sex within a single population”. When competitive between the dominant and the sub-dominant males, the ‘sneak’ strategy is one of most common alternative mating among several populations within animals which is basically characterised by speed. It is common that males who use sneak mating as an alternative strategy are comparatively small and do not present large fighting structures (Ra’anan and Sagi, 1985; Shuster, 1987; Clark, 1997).

In several animal cases, individuals practice only a single reproductive strategy throughout their lifetimes but alternative mating patterns are part of a developmental sequence during a single lifetime (Dominey, 1980). The sub-ordinate males are adopting an alternative tactic to gain access rapidly to mate with receptive females without courting. This approach has been observed not only in insects (Alcock *et al.*, 1977; Ward, 1983) but also in several species of crustacean such as helmet crab, *Telmessus cheiragonus* (Kamio *et al.*, 2003), the spider crab, *Libinia emarginata* (Laufer *et al.*, 1992; Laufer and Ahl, 1995), the snow crab *Chionoecetes opilio* (Sainte-Marie *et al.*, 1997), the American lobster *Homarus americanus* (Waddy and Aiken, 1990), the rock shrimp *Rhynchocinetes typus* (Correa *et al.*, 2003; Correa and Thiel,

2003a; Hinojosa and Thiel, 2003), the marine amphipod crustacean *Jassa marmorata* (Clark, 1997), *J. falcate* and *Microdeutopus gryllotalpa* (Borowsky, 1984, 1985a) and marine isopod, *Elaphognathia cornigera* (Shuster and Sassaman, 1997; Tanaka, 2003).

1.9 Mating and moulting stage

In many crustaceans there is a clear relationship between the moult cycle, especially females, and mating behaviour. Throughout their lives, crustaceans usually undergo periodic moulting to accommodate growth and reproduction. In crustaceans, such as lobsters, shrimp, isopods, and crabs mating occurs soon after the female discards her exoskeleton (moult) while her shell is soft (Hartnoll, 1969). Early studies, Templeman (1934, 1936) showed that the best mating time for the American lobster, *H. americanus*, was when the female had just moulted.

1.10 Mate selection and sexual attraction behaviour

Sexual attraction is an early step of mating behaviour in crustacean. Both males and females are playing an active role in mate choice. The literature shows contradictory findings about which gender is the attractor and which one is the seeker. More in details about such contradictory will be illustrated below. For example, Schone (1961) points out that the male crustacean is usually the active (seeker) sex partner, although special activity on the part of the female has occasionally been observed. While in some crustacean species, the female is the seeker. Further examples of will be demonstrated below.

1.10.1 Female as an attractor

It has been reported by observations (in field and laboratory) that it is always the pre-moulted females that is the attractor to the male American lobster, *H. americanus* (Atema and Engstrom, 1971; McLeese *et al.*, 1977; Atema *et al.*, 1979; Cowan and Atema, 1985, 1990; Atema, 1986; Atema and Cowan, 1986; Karnofsky *et al.*, 1989; Karnofsky and Price, 1989; Cowan, 1991; Bushmann and Atema, 2000). Furthermore, there is some evidence that females at inter-moult stage are sexually attracting males of *H. americanus* for courting purposes (Atema and Engstrom, 1971). In some crab species, males were attracted to pre-moult females of the large edible crab, *Portunus sanguinolentus* (Ryan, 1966). The spider crabs *Inachus phalangium* and *I. dorsettensis* males were attracted to the post-moult females that have ripe ovaries and are about to lay their eggs (Diesel, 1986; Jones and Hartnoll, 1997). A male king crab, *Paralithodes camtschatica*, searches out a female that he senses is preparing to moult (Idyll, 1971). Others observed that females of shore crab *Carcinus maenas* were directly approached by males (seeker) (Eales, 1974; Bamber and Naylor, 1997; Sneddon *et al.*, 2003). Similarly, it was found in the blue crabs, *Callinectes sapidus* where the female is attracting the dominant males (Gleeson, 1991; Bushmann, 1999). Also, Bauer (1976, 1979) and Bauer and Abdalla (2001) showed that the recently moulted female shrimp of *Hepatcarpus paludicola*, *H. pictus* and *Palaemonetes pugio* had a very high level of attractiveness (95%) to males. Wigginton (2005) reported that during mating, the male of the crayfish *Orconectes propinquus* seeks out receptive females. Using a Y-maze experiment, Mathews (2003) concluded that males of *Alpheus angulatus* were attracted to pre-moult females. Moreover, Borowsky *et al.* (1987) reported that females of the amphipod crustacean *M. gryllotalpa* are attracting males.

1.10.2 Male as an attractor

In contrast, there are some cases where the male is playing an attractor role. In fiddler crabs, *Uca tetragonon*, males attract females (ovigerous and non-ovigerous) into their burrows for copulation. However, males accept females with late ripe stage eggs (Goshima *et al.*, 1996; Murai *et al.*, 1996; Nakasone and Murai, 1998). Díaz and Theil (2003) reported evidence of female rock shrimp *R. typus* looking for dominant males during mating season.

1.10.3 Mate preference in the freshwater prawn

Mating selection for the freshwater prawn, as in many crustacean species, is also controlled by females' moulting stage and social hierarchy of the males' morphotypes. Kamiguchi (1972) stated that male of the freshwater prawn, *Palaemon paucidens*, showed sexual attraction only to ripe newly-moulted females. Another freshwater prawn *M. australiense* was observed that at early courtship, the female frequently approaches the larger male (Ruello *et al.*, 1973; Lee and Fielder, 1982). A study conducted by Thomas (1998) who built a Y-shape design bioassay from opaque perspex dividers placed inside a fibreglass rectangular tank (80 x 75 cm) with static water. He found after 90 minutes of observation, that females of *M. rosenbergii*, at different maturation stages of their ovaries, were attracted to the BC males to different degrees. The question as to which gender of *M. rosenbergii* takes the initiative in sexual attraction and mating behaviour remains ambiguous and needs more investigation.

1.11 Social hierarchy in *M. rosenbergii*

A single age population of *M. rosenbergii* is characterized by a sex-associated size distribution in which the size of females are quite homogeneous and males show heterogeneous individual growth (HIG). Social interaction between individuals within a prawn population, especially between males, is one of the major factors which are causing the HIG phenomena in which forming a wide positively skewed distribution (Smith *et al.*, 1978; Brody *et al.*, 1980; Cohen *et al.*, 1981; Ra'anan and Cohen, 1985). The differences between individuals, especially male morphotypes, are clear enough but the question must be asked as to how these differences arise. Various hypotheses have been advanced.

1) Competition for feed sources where the most aggressive and dominant individuals could suppress the smaller individuals (Segal and Roe, 1975; Karplus, 2005).

2) Appetite suppression in sub-ordinate individuals could result from agonistic interactions behaviour of social hierarchy among prawn individuals. In this circumstance, sub-ordinate individuals may grow less rapidly in spite of feed presence (Cobb *et al.*, 1982; Karplus, 2005).

3) Reduction in feed conversion efficiency where the smaller sub-ordinate prawn may have less efficiency in feed conversion (Karplus *et al.*, 1992; Karplus, 2005).

Within *M. rosenbergii* populations, the social hierarchy controls the behaviour of both males and females are not only on food and territory but also on reproductive activities. There is a strong and complicated social structure in which males play the most effective role. The body size factor has a substantial effect in social hierarchy ranking. In a comparative study of domination between the BC and the OC, Barki *et al.* (1991) found the largest chelae male (usually BC) in a mixed population of BC and OC males

is dominant over all OC and over all the smaller BC males as well. Moreover, Karplus *et al.* (2000) found that the BC males dominated OC males, irrespective of their size. This dominant male obtains advantageous access to food resources. Also, it obtains larger, better territories and is more likely to sequester sexually mature females for mating (Ra'anana and Cohen, 1985). Growth and sexual maturation variation among male morphotypes of *M. rosenbergii* are directly influenced by social structure within a population (Ra'anana and Cohen, 1984; Barki *et al.*, 1991; Karplus *et al.*, 1991, 1992).

However, the number of individuals in a certain area (stocking density) may also have a considerable influence in shaping the social structure of *M. rosenbergii* populations. Karplus *et al.* (1991) tested both male and female prawns for 84 days at a sex ratio of 1:1. Prawns stocked into either large cage (4.0 x 3.0 x 1.0 m) (volume of 12 m³) for communal culture (24 males and 24 females) or small (0.5 x 1.0 x 1.0 m) cages (around 0.50 m³) for paired culture (one male and one female). They found that 9 out of 10 OC males transformed into BC in the large cages, 8 of which (OC) became larger than all the BCs present. On average, Karplus *et al.* (1991) found that the newly transformed BC was larger 35 - 40% than the largest BC already present. In contrast, in stocks of single males kept into small cages, 60% of OC transferred into BC males. They suggested that stocking density may play a role in controlling interactions between the BC and OC males.

The large males could play a regulatory role in this social hierarchy. Malecha (1977) found the presence or absence of large males (BC or/and OC) greatly modifies the growth of SM males. Ra'anana and Cohen (1985) reported that the presence of either BC

or OC males suppresses the growth but not the maturation of the SM. It is worth mentioning that the dominance of BC over the other male morphotypes does not last continuously. Fujimura and Okamoto (1972) suggested an involvement of social interactions in growth regulation when the large prawns were removed from the population and the remaining small prawns gained further growth. In addition to the Ra'anand and Cohen (1985) study, a further study by Barki *et al.* (1991) noticed that when males were raised in a mixed gender group, only some of the SM males will transform into OC and eventually into BC while the rest will remain SM. When males are grown in isolation, Barki *et al.* (1991) demonstrated that a juvenile will transform first into a small male (SM), then into an OC male and finally into a BC male. When the fast-growing individuals were separated from the slow-growing prawns, Malecha (1977) found that the latter group resumed a more rapid rate of growth. Thus, the presence of BC males is likely suppressing the transformation of OC males to BC. The OC male transformation to the BC male occurs after becoming larger than the largest BC male in its vicinity, either by displacing a smaller or weaker BC male or in the case of BC male(s) death (Ra'anand and Cohen, 1985). The replacement pattern of BC by OC is termed a "leapfrog" growth pattern (Ra'anand and Cohen, 1985). This leapfrog growth pattern results in the gradual decline in the social position of the original dominance of BC males.

1.12 Size variation

Size variation phenomenon occurs in many animal species. In some freshwater prawns, the literature shows the existence of size variation among individuals within a population whether between conspecific or between opposite genders. For instance, size

variation has been observed in different prawns' populations such as *M. idea* (Thampy and John, 1973), *M. australiense* (Lee and Fielder, 1983), *M. hainanense* (Mantel and Dudgeon, 2005) and *M. rosenbergii* (Sandifer and Smith, 1975).

In *M. rosenbergii* populations, although the newly metamorphosed post-larvae are homogeneous in their size, it has been reported that the size distribution changes gradually with time as both variance and skewness increase (Sandifer and Smith, 1975; Ra'anan and Cohen, 1985). Within two months post-metamorphosis, the fast growing individuals could be observed which called "jumpers" and can grow as much as fifteen times larger than the rest of other individuals (Willis and Berrigan, 1977). While the "laggards" are considered slow growing individuals. However, when individuals reach sexual maturation, the size frequency distribution becomes clearer among individuals especially in male morphotypes (Ra'anan and Cohen 1985).

Few studies have been conducted to investigate the growth performance of prawn in isolation and communally. Ra'anan and Cohen (1984) found the average weight of juvenile *M. rosenbergii* cultured communally is higher than those cultured individually. Also, when juveniles of *M. australiense* were cultured communally, Lee and Fielder (1983) found more biomass than those reared individually for the same period of time and under similar laboratory conditions. However, in the two previous studies, the animals reared individually were more uniform in their growth than those that were reared communally.

It has been reported that a complex of several factors are involved in size variation within crustacean populations. Those factors, mainly, are associated with:

(a) Environmental factors where could cause competition for food and space (Segal and Roe, 1975; Cobb *et al.*, 1982; Pierce and Law, 1982; Karplus *et al.*, 1986; Karplus, 2005).

(b) Inter-related social factors, such as social status and territoriality, position within the size hierarchy (Ra'anana and Cohen, 1985; Barki *et al.*, 1991, Barki *et al.*, 1992; Karplus, 2005).

(c) Intrinsic factors, such as hatching and/or metamorphosis order (Smith *et al.*, 1978; Sandifer and Smith, 1979; Karplus *et al.*, 1990; Kulesh and Guiguinyak, 1993).

(d) Genetic differences (Malecha, 1977; Malecha *et al.*, 1980; Malecha *et al.*, 1984).

Adult male morphotypes of *M. rosenbergii* not only differ in their morphological characteristics, as described earlier, but also in their behaviour patterns (Brody *et al.*, 1980). Ra'anana *et al.* (1991) reported that the SM, which have small chelae, grow slowly and exhibit a normal distribution and represent around 50% of the adult males. The OC male morphotypes which are relatively larger than the SM, having larger claws, grows rapidly are highly variable in size and represent about 40% of the adult male population. The BC male morphotype represents around 10% of the adult male population, and are highly variable in size and grow slowly. Such difference between male and female growth strategy strongly influences the marketable yield. This tends to have greater size variation at harvest. Thus, size variation in *M. rosenbergii* population is a major constraint in commercial production. The key to the control or manipulation

of prawns' size variation lies in understanding the population structure. Several approaches have been suggested to increase their mean size and uniformity.

1.13 Size control

The natural complex social structure of *M. rosenbergii* plays a major factor in limiting expansion of culture (D'Abramo *et al.*, 1991). As previously mentioned, the HIG is mainly caused by the influence of the male morphotypes, which are controlled by the dominant males, especially the BC, within a population by depressing the growth of the other individuals. Since the prawn market is highly dependant on weight and size, this size disparity is causing management and production obstacles at harvesting. Hence, the presence of large quantities of undersized small male prawns can make the culture uneconomical. The potential for successful culture of *M. rosenbergii* lies in standardising the stock size and applying proper management procedures both prior to and after stocking. Various strategies have been investigated involving either manipulating management or/and certain environmental conditions.

1.13.1 Environmental conditions manipulation

Generally, metabolic rate is directly related to water temperature, increase by increasing temperature. Temperature could reduce HIG indirectly by diverting energy toward somatic growth more than to sexual maturation. In a comparison study of culturing *M. rosenbergii* in two different water temperature locations (25°C in Kentucky, and 29°C in Mississippi, USA), with similar variables such as stocking rate, stocking density, diet and feeding rate and source of juveniles, Tidwell *et al.* (1994) found significantly, fewer prawns reached sexual maturity at the 25°C site than those at the 29°C site. Tidwell *et*

al. (1994) suggested that the delay in sexual maturity at the 25°C site may lead to more energy being directed to somatic growth with less to maintenance and reproductive activities. They suggest a possibility that low water temperature (25°C) might have a considerable impact on population structure with potentially positive implications for production.

Also, the average stocking density size could play a certain role in production rates of prawn. Ra'anand Cohen (1984) showed the average growth rate, the level of size variation and the degree of skewness are directly related to the density of the newly metamorphosed post-larvae when tested at various stocking densities. D'Abramo *et al.* (1991) illustrated that when they stocked prawns weighing 0.75 g compared to 0.17 g at a stocking density of 40000 ha⁻¹ production was increased by 29%. Although production increased, the individual average weight at harvest decreased, however, manipulating the environmental conditions may increase yield production, the size variation within a population still remains one of the major problems in *M. rosenbergii* commercial mass production.

1.13.2 Selective harvesting

Selective harvesting by removing the large BC and OC and large females is another technique to enhance the growth of the remaining small prawns (Malecha, 1983). This technique was introduced first by Malecha (1981) (cited in Malecha, 1983a) who gave the term “traditional system” or “continuous culture ” to prawn culture technique in which stocking the post-larval prawns takes place once or twice a year into earthen ponds and then the market-sized individuals are harvested selectively using seine nets.

This system was designed in Hawaii by Fujimura (1974) to take advantage of the variable growth rates of individual prawns by culling the large ones (market-size, usually OC and BC males) from the pond by the nets and leaving the smaller prawns in the pond to give them more opportunity to grow on throughout the year. One more advantage of this technique is that selecting large animals results in reducing the competition for resources (food, oxygen and shelter). This method has also been practiced by Cohen *et al.* (1983b); Ra'anan and Cohen (1983) who found by applying this method throughout the grow-out season results in the SM males promptly transforming into OC males and shifting into the rapid growth pattern. Thus, the percentage of marketable males at the end of the period was significantly improved.

1.13.3 Size-grading

The size grading and re-stocking of prawns is based on compensatory growth of small individuals after removing the larger ones within a prawn population. To replace the traditional continuous stocking and harvesting system, Malecha *et al.* (1983a) suggested a multi-stage rotational stocking and harvesting system for year-around prawn monoculture. Ra'anan and Cohen (1983) reported an improvement in the average weight, yield and food conversion ratio (FCR) in the more frequently graded more than the non-graded stock. D'Abramo *et al.* (1991) conducted a preliminary study on freshwater prawn *M. rosenbergii* size-grading in monoculture combined with stock rotation, they found an increase in prawn mean size and yield and a decrease in the time from stocking to harvest. Moreover, Karplus *et al.* (1986b, 1987) demonstrated that size-grading in *M. rosenbergii* can have a considerable influence on the male morphotypes. Karplus *et al.* (2000) suggested that determination of the early male developmental pathway is different among the prawn male morphotypes.

Some of the size-grading studies of *M. rosenbergii* were applied by dividing a population of juveniles into two (or more) main fractions. However, when a population is split into two fractions of *M. rosenbergii* juveniles the upper (large) and the lower (small) sizes and stocked at the same density, it was found that the upper fraction juveniles subsequently developed into the large OC and BC at a higher frequency than the lower fraction (Ra'anana and Cohen, 1983; Karplus *et al.*, 1986b, 1987; D'Abramo *et al.*, 1991; Daniels and D'Abramo, 1994). Furthermore, after a grow out season of 97 days, Karplus *et al.* (1987) found the income obtained from the upper fraction prawns was nine times greater than the lower fraction prawns.

1.13.4 Comments about the selective harvesting and size-grading

Although the selective harvest and size-grading types of management have shown some improvement in yield production and production management, they also have some disadvantages:-

- 1) Both of these types of management are followed by draining the pond until the remaining biomass become small enough to consolidate stock with that of other ponds (Malecha *et al.*, 1987). Consequently, this practice is expected to incur extra operational and labour costs.
- 2) Usually, selective harvests are practiced in tropical climates where water temperature is adequate around the year but impractical in sub-tropical climates.
- 3) With both selective harvesting and/or size-grading, however, it cannot be certain that all the large animals have been removed during the process of netting prawns.

Consequently, some large animals may still remain to utilise resources and more importantly to suppress the growth of smaller individuals.

4) It has been reported that the size-grading technique, as applied by using seine nets is labour-intensive, time-consuming, and inefficient and can exacerbate harvesting losses (New, 1990).

5) These methods require skilful workers, which make it inapplicable under large-scale conditions (Brown, 1991; Karplus *et al.*, 2000).

6) It increases stress and mortality and adversely affects growth (Lam and Wang, 1987).

In general, these methods, both selective harvesting and size-grading, do not fully-solve the problem of the HIG, a major commercial problem in *M. rosenbergii* production.

1.13.5 Mono-sex culture

An alternative means of minimizing the HIG and maximizing yield of *M. rosenbergii* can be attained by growing a mono-sex culture. The technique of mono-sex or sub-population production has been applied for many years to many farm animals (e.g. the poultry industry, cattle, fish, and also, applied more recently to crustaceans such as red claw crayfish, *Cherax quadricarinatus* (Curtis and Jones, 1995). Applying this technique to *M. rosenbergii*, however, has involved different approaches taken with differing results. One is to concentrate on producing the faster-growing sex, the males, while others focus on the more homogeneous production of females.

In an outdoor concrete tank (3.0 x 2.0 x 1.0 m) in Saudi Arabia, a mono-sex culture of *M. rosenbergii* was investigated by Siddiqui *et al.* (1997). They found that all-male stock showed better growth and FCR than both all-female and mixed-sex populations. Under cage culture conditions, one of the early attempts of mono-sex culture was carried out by Sagi *et al.* (1986). In their trial, they stocked 40 juveniles with an average weight of 6.57 g in 2m³ cages and they found that the all-male *M. rosenbergii* population yielded 473 g m⁻² compared to 260 g m⁻² and 248 g m⁻² for the mixed and the all-female population, respectively after a 152 day growout period. Selective harvests were held fortnightly to remove any prawns weighing 30 g or more. By the end of the trial, they found that 70% of the males in the all-male group reached market size (30 g), while of the males in the mixed populations only 40% reached the market size. In comparison, the females reaching market size from the all-female group was 51% while in the mixed population group only 19% reached market size. They concluded that culturing all-males will lead to a shorter culture period. However, it is worth mentioning that this experimental work was conducted in a small water body (not in a large scale) and under very intensive conditions with periodic selection (every two weeks). The survival rate of the all-male group was somewhat low (76.5% for all-male and 86.5% for all-female). Studies on mono-sex culture in *M. rosenbergii* highlight the differences in growth between males and females but still do not eliminate (prevent) the HIG. Nevertheless, these studies have lead to interest in producing mono-sex offspring from sex reversed prawns.

1.13.6 Polyculture

Cohen *et al.* (1988) investigated the potential of culturing *M. rosenbergii* juveniles (2.6 g) in earthen ponds at stocking densities of 9 m⁻² as a mono-sex culture along with

common carp at a stocking density of 500 ha⁻¹. At the end of the 150 days experimental period they found the all-male production (2,200 kg ha⁻¹) was higher than the all-female and the mixed sex populations (1,640 kg ha⁻¹ and 2,041 kg ha⁻¹, respectively). They also found that the average weight of marketable (> 25 g) prawns in the all-male population was 36.9 g while average weight of 24.1 g and 30.1 were found in the all-female and the mixed sex population respectively.

Other mono-sex culture studies integrated with other freshwater fish species have been conducted. In an experiment in earthen poly-culture ponds consisting of common carp, male tilapia and silver carp along with all-male, all-female and a mixed gender (1:1) of *M. rosenbergii* juveniles (2.6 g average) for period of 120 days at stocking density of 1.25 m⁻² was conducted by Hulata *et al.* (1988) who found the survival rate of all-female *M. rosenbergii* was 90% and the all-male group was 85%. They found also that the total production in the all-male group was significantly higher than all-female group (600 kg ha⁻¹ and 471 kg ha⁻¹, respectively) but not significantly different from the mixed sex (554 kg ha⁻¹). Moreover, the net income from the all-male prawns was 18% higher than the control (mixed gender) whereas the net income from all-female prawn was 25% lower than the control. Hulata and co-workers had sexed the *M. rosenbergii* population manually prior to stocking for their experiment. Manual sexing is simple, easy and has been implemented earlier as in Hulata *et al.* (1988), but application of this method has some disadvantages. First of all, it has not yet proved possible to identify males under 3.5 cm total length (Janssen, 1987). Also, sexing requires not only high accuracy in sex determination of prawn individuals but also, it seems impractical especially ahead of the sexual maturation period. Although, Hulata *et al.* (1988) showed

a yield increase of 18% in net income via the all-male stocking strategy, this study concluded that the improvement in income was not large enough to justify the implementation of manual sexing. Thus, for large scale operation, this technique is impractical from a commercial point of view. In Brazil, Dos Santos and Valenti (2002) showed that stocking densities of up to 6 m⁻² *M. rosenbergii* PL did not affect the production of Nile tilapia (*Oreochromis niloticus*) stocked at 1 m⁻² over a 175-day experimental period, neither required additional feeding nor significant changes in management.

1.13.7 Sex reversal

Sex reversal has been used in many fish culture species such as pink salmon, *Oncorhynchus gorbuscha* (Funk *et al.*, 1973), coho salmon, *Oncorhynchus kisutch* (Hunter *et al.*, 1982), chinook salmon, *Oncorhynchus tshawytscha* (Hunter *et al.*, 1986; Baker *et al.*, 1988), common carp, *Cyprinus carpio* (Ali and Rao, 1989), and black porgy, *Acanthopagrus schlegeli* (Chang *et al.*, 1994). Also, this technique has been studied in some species of crustacean such as *Armadillidium vulgare* (Katakura, 1960, 1961; Hasegawa and Katakura, 1985; Suzuki *et al.*, 1990; Suzuki and Yamasaki, 1991), greasyback shrimp, *Metapenaeus ensis* (Yano, 1985), *Penaeus japonicus* (Yano, 1987), the crab *Eriocheir japonicus* (Lee *et al.*, 1993), ghost crabs, *Ocypoda platyarsis* (Sarojini *et al.*, 1962) and red swamp crayfish, *Procambarus clarkii* (Sarojini *et al.*, 1995; Nagamine and Knight, 1987b). Although this approach is not modern and has been applied in many species of crustaceans, research on *M. rosenbergii* is relatively limited. Production of either an all-female population or an all-male population with the elimination of male morphotypes should reduce the magnitude of size variation and contribute to higher yields.

Generally, a surgical technique is used for sex reversal in crustaceans. This is can be applied by micro-surgical either by ablation of the androgenic gland from the young males or implantation of androgenic gland tissues into young females. Ablating the androgenic gland of males of freshwater prawns, *M. rosenbergii*, at an early stage of development have diverted them to females “neofemale” (Nagamine *et al.*, 1980a). Based on two crosses (producing a total of 567 offspring) Sagi and Cohen (1990) showed that the mating of the neofemale *M. rosenbergii* with the normal male resulted in 99.1% and 100% male offspring.

Recently, the bilateral removal of androgenic glands of a total of 87 *M. rosenbergii* post-larvae at stage I (presence of the gonopore complexes but not the appendix masculina) resulted in 80.4% survival (Rungsin *et al.*, 2006). Eight out of 12 matured neofemales (Rungsin *et al.*, 2006) produced all male offspring. Aflalo *et al.* (2006) proposed a two-step scheme for large scale microsurgical sex-reversal (andrectomy) to overcome the difficulty of identifying males of *M. rosenbergii* at a sufficiently early stage of development. Briefly, these two steps are: phase I where 1904 post-larvae were andrectomised at 25 – 60 days after metamorphosis (PL_{25 – 60}) to produce neofemales that are capable of mating with normal male and spawning. Result obtained from this phase was only 1.28% successful. Subsequently, they introduced phase II which was conducted to andrectomise those presumed males obtained from the phase I at the earlier ages (PL_{20 – 30}). From phase II, Aflalo *et al.* (2006) found that 729 out of 4137 andrectomised male PL (17.6%) were suspected to be neofemales. Although their results have shown the potential of the technique for producing all-male offspring of *M. rosenbergii* for aquaculture, more information is needed because it is likely that sex

determination of freshwater prawn may not completely rely on the ZW system (Malecha *et al.*, 1992).

In contrast, Malecha *et al.* (1992) obtained a “neomale” (ZW) of *M. rosenbergii* by surgical implantation of androgenic gland tissues from adult males into very young putative females (6.5 – 7.5 mm CL, approximately 30 days after metamorphosis). When they mated these neomales with normal females, the offspring produced from 15 progenies had a sex ratio of 1:3.20 (male: females) which is significantly more skewed towards females than the primary sex ratio of 1:1. Furthermore, when female offspring from neomale parental crosses, a sex ratio of 1:6.63 was produced from 8 progenies and all females ($n = 1809$) was produced from one brood. Mortality rate from surgical androgenic gland implantation was high (89.5%) compared with the non-surgically manipulated control groups (48%). Surgical experience and techniques of andrectomy need to be improved. The age at which these surgical procedures are carried out can be crucial.

Administering sex hormones to young *M. rosenbergii* is an alternative approach for sex-reversed practice. Baghel *et al.* (2004) reported a significant difference in sex ratio of *M. rosenbergii* larvae fed *Artemia* nauplii enriched with lipid-containing 17 α -methyl testosterone (MT) mg^{-1} dry weight at 5, 15, 30, 50, and 100 mg l^{-1} . *M. rosenbergii* larvae were fed hormone-enriched *Artemia* nauplii for 50 days. Their findings, however, showed that the sex ratio is less than 0.93 skewed to female with any treatment concentration. Furthermore, when a diet prepared by mixing different doses of MT and fed to post-larval *M. rosenbergii* for 20, 40, or 60 days, Ohs *et al.* (2006) found no

significant changes were obtained of using this hormone since the sex ratio remained unchanged compared with the normal.

Thus, the sex reversal approach looks has its limitations in minimising the HIG. Further investigation and research in size variation to minimise or eliminate the HIG variation in *M. rosenbergii* and then maximising the yield production is required. In other words, it is important to study the reason(s) behind the HIG, especially among male morphotypes. This leads toward most focus to the social hierarchy aspect and how communication between individuals is associated with HIG.

1.14 Communication

Communication as defined by Wilson (1975) is an action by an organism that alters the probability of behaviour in another organism in an adaptive fashion. Communication, usually occurs when information is exchanged between a sender and a receiver, resulting in an alteration of behavioural pattern for one or both of the individuals (Enquist 1985). Bradbury and Vehrencamp (1998) argue that information exchange must benefit both the sender and the receiver to be called communication. Communication also can be defined as an “*interaction between a signaller*”, which produces a sensory stimulus or signal, and a receiver, who perceives the signal and makes a consequent behavioural decision” (Rosenthal and Lobel, 2006). Moreover, they reported another definition of communication. “*Communication is fundamentally a series of steps between one animal’s brain and another: from the production of behaviours and strategies on the signalling end to perception and behavioural response on the receiving end*” (Rosenthal and Lobel, 2006). The surrounding environment for

aquatic crustaceans' population is one of the most important factors in communication. Communication in an aquatic environment generally presents an instructive comparison to communication in terrestrial habitats (Rosenthal and Lobel, 2006). Studying communication within the aquatic environment is also difficult.

Rosenthal and Lobel (2006) illustrated the difference between the signal and stimuli. One of the practical challenges of communication in such types of animal is distinguishing between a signal (a stimulus that has evolved in an explicitly communicative context) and a cue (a stimulus whose communicative function is incidental). Signals are easy to identify if they involve behaviours performed only in the presence of an appropriate receiver; many acoustic calls and motor displays fall into this category. Other stimuli, like sex hormones released during courtship, are more difficult to classify. The situation is further complicated by the fact that some communication signals may have evolved in order to enhance transmission of a cue to a receiver. The social interaction relationship among the individual of *M. rosenbergii*, especially within male morphotypes, is mediated by visual, tactile and chemical cues.

1.15 Types of communications

It has been reported that contact in crustaceans occurs when males and females touch each other during courtship and mating (Salmon, 1983; Borowsky, 1991; Kelly *et al.*, 1998; Correa and Thiel, 2003b). These communication cues are usually mediating agonistic behaviour, reproductive, and social hierarchy of aquatic crustacean. Often both genders are involved in signal exchange (Atema and Voigt, 1995; Bushmann, 1999). In other words, when individuals assess each other, they may base their decision

on more than one signal (Sneddon *et al.*, 2003). This is expected during sexual attraction behaviour in those species where members of one or both sexes show strong preferences for specific individuals of the opposite sex. Several studies showed different findings in examining sexual communication in crustaceans exclusively on either visual cue (Marshall *et al.*, 1999), tactile (Daniels, *et al.*, 2000) or chemical cue (Juarez *et al.*, 1987).

1.15.1 Tactile communication

Obviously, tactile contacts in crustaceans usually occur within short and close distance between individuals. Tactile communication between crustacean individuals of the cohort can be presented in different patterns of signals such as body rises and chelae waving (Solon and Cobb, 1980), antennae waving (Rubenstein and Hazlett, 1974; Itagaki and Thorp 1981; Bruski and Dunham, 1990), and a flicking motion by the antennules (Schmitt and Ache, 1979). Signalling can include behavioural acts by one animal that alter the subsequent behaviour of the receiver. The use of the antennules, crustacean chemosensory organs, in agonistic interactions has also been well documented (Rutherford *et al.*, 1996; Smith and Dunham, 1996). The flagella of American lobster *H. americanus* are also brandished vigorously during agonistic encounters between conspecifics (Atema and Cobb, 1980). Moreover, tactile communication in crustacean can occur in different light conditions.

1.15.2 Visual communication

The visual signal is very important for information exchange and defence among crustaceans. Visual sexual communication in crustaceans involves cues such as

morphological structure, shape, colour and shelter size (Latruffe *et al.*, 1999; Christy *et al.*, 2003). Studies on different crustacean species showed the importance of visual signals in studies including hermit crabs, *Pagurus marshi* (Hazlett and Bossert, 1965; Dunham and Tierney, 1983), fiddler crabs, *Uca pugilator* and *U. pugnax* (Hyatt and Salmon, 1979); Zeil and Hemmi, 2006), crayfish, *O. rusticus* (Bruski and Dunham, 1987), spiny lobster, *P. argus* (Wilkens *et al.*, 1996) and the freshwater prawn, *M. rosenbergii* (Juarez *et al.*, 1987). Visual communication among crustacean individuals depends on light. Bruski and Dunham (1987) observed that the frequency of communication among the crayfish, *O. rusticus* by visual cue decreased in the absence of light while the tactile behaviours were increased.

1.15.3 Chemical communication

In the aquatic environment not only visual-signals and tactile cues are used but they are also accompanied by chemical cues (Salmon, 1983). Chemical cues serve as a fundamental source of sensory information linking a variety of interacting organisms (Dusenbery, 1992; Dodson *et al.*, 1994; Burrs and Lodge, 2002; Zimmer-Faust and Butman, 2000; Zimmer, 2000). Others, Bradbury and Vehrencamp (1998) for example, reported that chemical communication involves the provision of information by the sender to a receiver and the subsequent use of that information by the receiver in deciding how to respond.

It has been reported that several crustacean species are combining all possible signals, including chemical cues, in their communication such as in the snapping shrimp *A. heterochaelis* (Hughes, 1996), in mantis shrimps (stomatopods) the fiddler crabs (genus

Uca) (Christy and Salmon, 1991; Marshall *et al.*, 1999), and in freshwater crayfish (Acquistapace *et al.*, 2002). In aquatic crustaceans, water is considered not only the habitat but also the carrier of chemical cues between individuals. Since physical and visual senses in water are restricted to limited distance due to water turbidity, however, chemical cues are an important source of information within a population to what occur to the terrestrial environment (Eisner and Meinwald, 1995).

Chemical cues are playing a considerable role in communication among individuals within a population such as identification for territorial and competition and for sexual attraction. Because chemical cue can easily broadcast and flow in the aquatic environment over both short and long distances, crustaceans might use this means largely for social signalling and breeding behaviour. Chemical communication has been shown to influence behaviour of various crustaceans (Ryan, 1966; Caldwell, 1979; Seifert, 1982). To investigate the mechanisms that regulate social behaviour interactions, especially mating and copulation strategies within crustacean populations, it is necessary to understand regulation of the appropriate interface between its internal physiological condition and the external surrounding environment conditions.

The literature contains different observations and suggestions about this type of communication among aquatic crustaceans. Research has demonstrated the importance of chemical communication cues in controlling the sexual attraction and copulation for some crustaceans such as shore crabs, *C. maenas* (Bamber and Naylor, 1997) and American lobsters, *H. americanus* (Bushman and Atema, 2000). Chemical cues allow crayfish to distinguish between male and female conspecifics (Ameyaw-Akumfi and

Hazlett, 1975; Dunham and Oh, 1996) and to identify the correct species in mate choice (Tierney and Dunham, 1982). Ameyaw-Akumfi and Hazlett (1975) discovered that male *P. clarkii* can distinguish females from males through a putative sex pheromone. Dunham and Oh (1996) reported the same behaviour in female *P. clarkii*. Díaz and Thiel (2004) concluded that there is a sexual communication in rock shrimp, *R. typus*, occurring between both the receptive female and the robustus (dominant) males which rely on chemical signals.

Juarez *et al.* (1987) suggested that chemical communication via water currents inhibited growth of *M. rosenbergii* juveniles and increased size variation in prawns reared under laboratory condition. Moreover, Ra'anan and Cohen (1985) suggested that social ranking among *M. rosenbergii* males is likely to be associated with the hormones of reproductive activities released in the water. Since *M. rosenbergii*, and many other crustacean species, are mostly nocturnal and naturally live in turbid environments which both can limit visual communication (Bovbjerg, 1970), they must rely increasingly on non-visual sensory input.

1.16 Pheromones

A pheromone is a chemical substance which is released from an organism into the environment where it serves as a message to others. Pheromones are widely used within the animal kingdom in a great variety of species and also include bacteria and plants. Such chemical substances or odours are playing a major role in regulating behaviour and social life of many animals including crustaceans, which are usually used for a range of functions such as sexual attraction, agonistic interactions and identification

signals. However, studies about pheromones on aquatic crustaceans are relatively having less extent.

In the aquatic environment, water is the only carrier of chemical cues for communication, including pheromones, between crustacean individuals. Sexual recognition and attraction which precedes the mating behaviour is stimulated by sex pheromone (Barth, 1961). The term 'pheromones' defined as 'substances which are secreted to the outside by an individual and received by another individual of the same species, in which they release a specific reaction (Karlson and Lüscher, 1959). Later, Sorensen and Stacey (2004) defined a pheromone as “an odour or mixture of odorous substances, released by an individual (the sender) and evoking in conspecifics (the receivers) adaptive, specific, and species-typical response(s), the expression of which need not require prior experience or learning”. Atema and Cowan (1986) used the term pheromone to describe any body odour produced with a role of communication, while ‘body odour’ in their terms referred to all chemical emissions from metabolism such as urine so this could also include a number of pheromones. Pheromone plays a critical role in the communication especially for reproductive activities in nearly all animals (Shorey, 1976). For instance, Kamio *et al.* (2005) reported that sex pheromones are crucial chemosensory cues that trigger and modify reproductive behaviours in conspecifics of helmet crab *T. cheiragonus*. In a bioassay system, Hardege *et al.* (2002) demonstrated that *C. maenas* male mate selection is based on the detection of female odour.

1.16.1 Source of pheromone

The literature agrees that a pheromone is a chemical substance involved in communication among aquatic Crustacean which affects their sexual and social activities. There is a debate as to whether it is males or females that are producing the pheromone. Furthermore, it shows that pheromone may contain different chemical cues in which some of them are associated with the females' moulting "ecdysones" while others relate pheromones to the urine.

1.16.2 Pheromone released by females

The existence of a female sex pheromone in crustaceans was demonstrated early in *H. americanus* (Hughes and Matthiessen, 1962) when they noticed a positive response of attraction behaviour to water collected from freshly moulted females. Since then, a list of several crustacean species for which there is evidence of releasing female sex pheromone has become extensive and continued to grow. Later, other studies, Cowan and Atema (1990) noticed that females orientate towards male burrows in response to sex attractants emitted by males and female-produced sex pheromones control mating behaviour.

Female's sex pheromone also exists in crabs which use it to attract males. Early, Ryan (1966) demonstrated the existence of chemical cues in water collected from tanks containing sexually mature pre-moult females of the edible crab, *P. sanguinolentus*. Hartnoll and Smith (1979) reported that in many crab species females including the edible crab, *C. pagurus* about to moult release pheromone into the water with her urine then mate immediately after moult. However, this pheromone is absent from inter-moult

females and immature pre-moult females. Meantime, pheromone is absent from the urine of the edible crab, *C. pagurus* males as Hartnoll and Smith (1979) reported. Later, other studies, Eales (1974); Seifert (1982); Bamber and Naylor (1996a) showed the existence of chemical cues in some other crab species involved in sexual attraction that released from female of shore crabs *C. maenas*. In the blue crab *C. sapidus*, Gleeson (1980, 1982, 1991; Gleeson *et al.* 1984) described the presence of a pheromone in female urine that was attractive to males.

American lobsters, *H. americanus* (McLeese *et al.*, 1970, 1973, 1977; Atema and Engstrom, 1971; Atema and Cowan, 1986) and crayfish, *P. clarkii* (Ameyaw-Akumfi and Hazlett, 1975). In helmet crabs *T. cheiragonus*, females advertise their reproductive status and attract males via water-borne chemical cues (Kamio *et al.*, 2000). Borowsky (1984) reported that the female the amphipod crustacean *M. gryllotalpa* secrete substance(s) which attract males while males do not secrete.

It has been suggested that during the moulting process, odours emission from females may considered a pheromone. Kamiguchi (1972) concluded that the soft shell of newly-moulted female freshwater prawn *P. paucidens*, is an important factor for provoking males into mating behaviour. Atema and Cowan (1986) reported that females of *H. americanus* showed consistently strong responses to male moulting odour more than female moulting odour. Atema and Cowan (1986) suggested that these chemical cues are either arising from the moulting hormone “crustecdysone“or/and to any metabolite substance. Bouchard *et al.* (1996) found males of snow crabs *C. opilio* significantly increased their activities and demonstrate sexual behaviour to water containing recently

moulted females. They attributed these kinds of activities to the influence of ecdysteroids which are likely released from the newly-moulted females and then used as a cue in the mating process. Similarly, in *C. maenas*, water-borne signals from the pre-moult female evoke searching in males (Bamber and Naylor, 1996a).

1.16.3 Urine released by female

Evidence, however, is scarce on how the urine pheromone in itself affects males. Hinsch (1968) reported that urine collected from the inter-moult females of spider crab, *L. emarginata* failed to demonstrate a sexual response from a receptive male. In contrast, many other studies on sexually mature females of some crustacean species have shown that urine is the main carrier of putative pheromone as studied in shore crabs *C. maenas* (Eales, 1974; Seifert, 1982; Bamber and Naylor, 1996a) and American lobsters, *H. americanus* (Atema and Cowan, 1986). These studies confirmed the existence of a sex pheromone that was released into the water body via urine which produced a positive response in the receptive male. A study conducted by Bamber and Naylor (1997) who reported that 17 out of 20 shore crabs, *C. maenas*, males exhibited a positive sexual response to pre-moult female urine. They confirmed that urine from the pre-moult female was a source of sex pheromone. Atema and Cowan (1986) reported that urine released from inter-moult females of *H. americanus* caused stronger responses than male urine in both sexes, whereas, females responded weakly to male urine. They suggested that male and female urine are chemically different and female urine may contain a higher volume of the same active fraction than male urine. Furthermore, Bushmann and Atema (1997) concluded that urine released from female *H. americanus* controlled male aggression but mating and restricting females' urine release reduced male aggression and facilitated mating. Similarly, Gleeson (1991) noticed that blue

crab, *C. sapidus* males showed positive sexual responses to the urine from ripe females while there was no positive response of female to urine from males.

1.16.4 Pheromone and urine release by males

There is some evidence that males are producing their pheromone via urine. It has been reported that male urine release is important for individual recognition in male agonistic interactions (Karavanich and Atema, 1998a; Breithaupt *et al.*, 1999). Furthermore, several studies showed that urine from male crustaceans has an influence in sexual attraction and mating. For example, in *H. americanus* (Cowan, 1991) and *C. sapidus* (Bushman, 1999), the female is guided towards the male by a pheromone in the male's urine. Another study conducted on *H. americanus* during courtship and agonistic interactions, Karavanich and Atema (1991) found males release urine that contains cues identifying themselves and conveying their presence to the opponents. Bushmann and Atema (2000) found that the urine male *H. americanus* contains compound(s) important for close-up mate evaluation. They expected that males' urine cues play a role in female identification of males and evaluation of male quality. Restricting (*i.e.* blocking) urine secretion, Bushmann and Atema (2000) found a significant reduction in the number of approaches and the time spent for females to enter the males' shelter.

1.17 Urine

Urine is considered not only as metabolic waste fluid product excreted from the crustacean body but also is carrying chemicals which may be involved in communication. Those chemical cues may consist of a unique or a mixture of substances produced from particular glands and released into the urine (Dunham, 1978).

Urine is stored in the bladder and released through a paired set of nephropores, small openings found on the ventral bases of antennae. Crustacean urine is released from these bilateral nephropores and by the aid of gill current, urine can broadcast forward for up to seven body lengths anteriorly (Atema, 1985), by the aid of powerful gills, maxillipeds, pleopods along with nephropore propulsion currents (McPhie and Atema, 1984; Atema, 1986; Breithaupt, 2001; Herberholz and Schmitz, 2001). Atema and Cowan (1986) pointed out that crustacean urine is stored in a bladder as an indication of the need of controlled release.

Urine might provide identification of species, sex, and perhaps social hierarchy status and individual identity. Many studies showed the roles of urine in communication between crustacean individuals in agonistic behaviour of crayfish *O. rusticus* (Zulandt Schneider *et al.*, 2001; Bergman *et al.*, 2003; Moore and Bergman, 2005), *P. clarkia* (Zulandt Schneider and Moore, 2000), *Astacus leptodactylus* (Breithaupt and Eger, 2002), American lobsters, *H. americanus* (Karavanich and Atema, 1991; Breithaupt and Atema, 1993, 2000; Karavanich and Atema, 1998a, b; Breithaupt *et al.*, 1999), rock lobsters *Jasus edwardsi* (Thomas *et al.*, 2003), and shore crabs, *C. maenas* (Sneddon *et al.*, 2000). In addition, different studies demonstrated that sexual behaviour and chemical attraction have strongly associated in a number of crustacean species such as crayfish *O. virilis* (Hazlett, 1985), *Pacifastacus leniusculus* (Stebbing *et al.*, 2003), American lobsters, *H. americanus* (Atema, 1986; Cowan, 1991; Bushmann and Atema, 1997), shore crabs, *C. maenas* (Bamber and Naylor, 1996a; 1997), and helmet crabs, *T. cheiragonus* (Kamio *et al.*, 2003; 2005). Since urine in many crustacean species is playing a significant role in carrying such chemical cues (pheromones) out into the

surrounding environment, several studies on both genders have focused on the influences of urine on sexual behavioural responses.

1.18 Antennal gland

It is well known that urine in crustacean released from the antennal gland. Decapod crustaceans have a bilateral pair of antennal (green or maxillary) glands which are located at the base of each antenna of the anterior ventral region of the cephalothorax. Each antennal gland (AG) has a single opening (nephropore) which is located on the underside of the coxae of the antennae (Vogt, 2002). One of the main functions of the AG in crustaceans is in regulating the water and ion contents between the internal body and the surrounding water medium as part of their “osmoregulation”. Also, the AG works as a tool to excrete the metabolic waste products outside the animals’ body. This organ, however, has been studied by many researchers in many crustacean species. Moreover, the AG in marine decapods is involved not only in control haemolymph volume but also involved in hyporegulation of magnesium and sulphate in the haemolymph, excretion of organic substances and reabsorbing of fluid, sugar and amino acids from the primary urine filtrate (Mantel and Farmer, 1983).

Chapter 2 Material and methods

2.1 General material and methods

Only details of those materials and methods commonly utilised throughout this study are given in this Chapter. Further details of materials specific to discrete sections of this thesis are given in each particular chapter.

The main aim of this thesis is to study chemical communication of *M. rosenbergii* particularly in relation to behaviour related to size variation. Behavioural tests were designed and applied using a bioassay tank as show in Figure 9.

2.2 Part I: Sexual behavioural responses

2.2.1 Stocking *M. rosenbergii*

M. rosenbergii were brought from Malaysia. Stocks of *M. rosenbergii* were held in 88 individual plastic tanks (45 x 35 x 25cm, L, W and H) within the Tropical Prawn Unit, the Institute of Aquaculture, University of Stirling. Each of these tanks was provided with a continuous running freshwater recirculation system, continuous aeration and a piece of 15 cm PVC pipe as a shelter. Water temperature of the system was maintained at $28 \pm 1^\circ\text{C}$ by using an electrical heater. Temperature was monitored daily. Around 20% of the water in the system was replaced daily with freshwater. The photoperiod was maintained at 12 h light/12 h dark. The other water quality parameters were carried out once a week such as $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NH}_3\text{-N}$ levels, (total ammonia was below 0.03 mg l^{-1}) pH was 7.2 – 7.6 and total water hardness was 120–180 mg l^{-1} . Prawns were fed daily to full satisfaction with chopped squid and green beans alternately and they were

maintained in these tanks throughout the course of the experiments trials when not being tested.

Each individual prawn used for these studies had a continuous moulting record pasted on the top of each tank showing their histories of date of moult, duration of moulting cycle (days) and carapace length (mm). Carapace length was measured by using a vernier calliper (to 0.1 mm) from the base of the postorbital margin to the mid-dorsal posterior edge of the prawns' carapace. Moulting records were used as a support tool to categorise prawns' moulting stages and help to judge the female's ovarian maturation stage. Based on classification reported by Chang and Shih (1995), ovarian development in female *M. rosenbergii* was classified into five stages according to the size and the colour of ovary observed through the external carapace. However, in the first experiment, females were categorised into three stages, the pre-moult, newly-moulted and inter-moult in which each stage was tested separately against the three male morphotypes.

2.2.2 Experimental system

The experimental system was built inside a wet laboratory, at the Tropical Prawn Unit, Institute of Aquaculture, University of Stirling. This system consists of the head tank which is high and big enough to provide heated and aerated freshwater to the experimental bioassay tanks. The experimental bioassay tanks were placed on the floor of the wet laboratory and the pipes and tubing to transfer water from the head tank to the experimental bioassay tanks.

2.2.3 The head tank setup

The head tank (HT) measured 1.5 x 1.0 x 0.90m (L, W and H) and held 700 litres of water when filled to a standpipe height of 0.5m. This tank was top-up with freshwater water when it is needed (usually twice a week). Three electrical heaters (VISI-Therm, 300 Watt each) and three air stones were submersed near the bottom of the HT. Water temperature was maintained at $28 \pm 1^\circ\text{C}$ and the dissolved oxygen level was 7.2 mg l^{-1} . Water fell from the HT at height of 1.6 m above the bioassay tanks and flows by gravity through one tube and then split into two equal and smaller tubing in which each is leading to one of the experimental bioassay tanks. Each of these bioassay tanks was built to test the chemical response of *M. rosenbergii* to conspecific scents.

2.2.4 Bioassay tanks setup

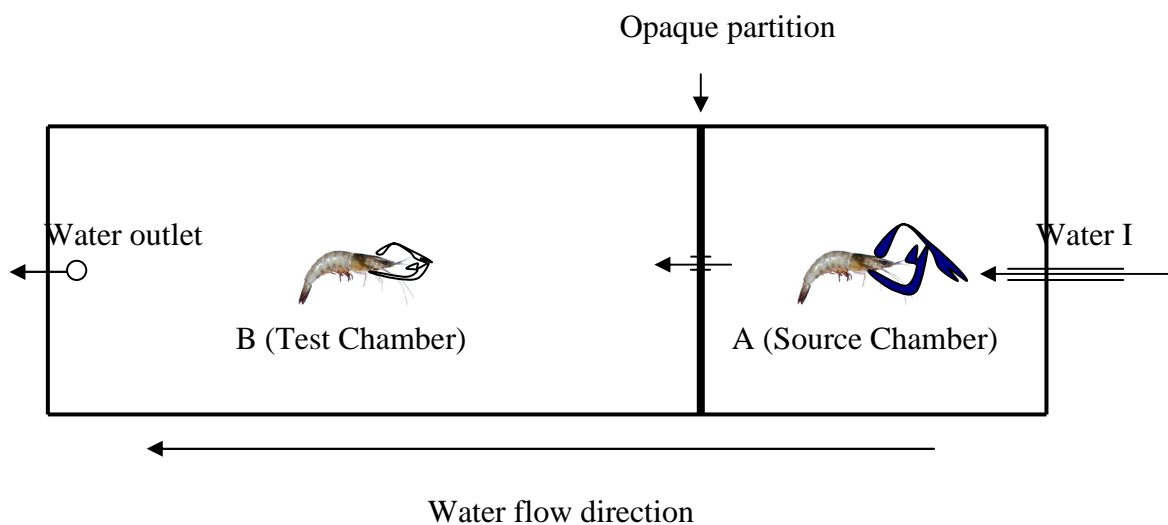


Figure 9: Plan view schematic diagram of the bioassay tanks system design.

The bioassay tanks system (Figure 9 above) is designed to test the attraction behavioural responses of the freshwater prawn *M. rosenbergii*. There were two rectangular opaque tanks which are completely opaque and each measured 1.10 x 0.50 x 0.18 m (L, W and H). Each of these tanks is considered as an experimental unit. At the far end of each tank there is a stand pipe to keep water level at 18 cm. Each tank was divided into two chambers (sections), the source and test chambers. The source chamber (A) measured 0.30 x 0.50 x 0.18 m (L, W, and H) and held 27 litres. Whereas, the test chamber (B) measured 0.80 x 0.50 x 0.18 m (L, W, and H) and held 72 litres. An opaque partition panel was constructed to be high enough not only separating the two chambers (A and B) but also precluding visual, tactile and water vibration contacts between the two experimental prawns and avoiding water overflow from the chamber A to the chamber B from both sides of the partition. This partition was mounted with aqueous silicon seal on all contact sides. There is one hole (3 mm diameter) at the centre of this partition wall allowing water to pass through from chamber A to chamber B. A constant flow of fresh water was maintained through the bioassay experimental tank at a rate of around 250 ml min⁻¹. Water flow is adjusted by a valve attached to each of these small tubing before entering the chamber A of the bioassay tanks. A drainage stand pipe was located at the central far end of the chamber B. For each trial, water runs only once and was never reused for another trial. Both of the bioassay tanks were placed and adjusted on the floor level prior to each trial.

2.2.5 Testing the efficiency of bioassay tanks

The whole system, tanks, valves, partition, hole, drainage etc. were tested at the beginning by running water through the entire system to ensure that system worked

efficiently and that there was no water leakage. A piece of fresh food (squid and/or green beans) was used to test the efficiency of the bioassay tank systems before running any experiment. Prior to running the behavioural trials, prawn was placed at the far end of chamber B and a piece of fresh food was placed inside the chamber A. Then the water valve was turned on to let water run into the system where prawns' behaviour responses were monitored. Usually, normal behaviour is considered when prawn remains at their place (far end close to the stand pipe) of the chamber B. Once the water flow was turned on prawns showed a positive response and went toward the central hole of the divider partition indicating that the prawns were able to detect the food odour and the bioassay system was working properly. This procedure was repeated several times to ensure the system was working properly. Also, to test the water flow from the chamber A to the chamber B via the central hole, a non-toxic aqueous ink (red) was injected in the chamber A and red water current was observed passing through the hole toward the chamber B and then to the drain stand pipe.

2.2.6 Behavioural observations

For testing the existence of chemical communication between prawn individuals, a receptive of sexually mature single prawn (male or female) was placed inside chamber B (close to the drain stand pipe), while an individual prawn to be tested for the possibility of producing chemical(s) cues was placed inside chamber A. Since there is only one hole in the divider partition, so water along with chemical cues had to pass through this hole going to chamber B. In order to get a short and reliable bioassay observation, preliminary experiments indicated that 30 minutes of observation for each trial is sufficient time for *M. rosenbergii* to establish a clear attractive behaviour. As a result, the positive behavioural response was considered if within 30 minutes the

receptive prawn moves from the far end of the chamber B (near the stand pipe) and approach (touch or get closer) to the central hole. While the behavioural response was considered negative when the time exceeded the 30 minutes and the receptive prawn did not move from their place toward the hole.

For the purposes of accuracy and documentary observation of sexual behavioural responses, a Closed-Circuit Videotape System (CCVS) was prepared. This CCVS is consisting of a digital video camera, a television (TV) and wireless transmitter. The digital camera brand is SONY (DCR-TRV460) which was sited on the top of the bioassay tanks to observe and record prawns' behavioural response. This camera is operated by a remote control device for distance control. In order to avoid any disturbance to the experimental prawns such as sound and movement during each behavioural trial, the digital camera was connected to a TV in an entirely separated area (dry laboratory next to the wet laboratory) by wireless video transmitter and receiver (THOMSON VS360) devices. In other words, the digital camera is used for dual purposes, for recording the prawns' behaviour and for a live transfer to the TV. Dim light was used and the intensity measured underneath water surface of the bioassay tanks which were 57 lux in all trials.

Prawns for testing were collected early in the morning from their holding tank in the main system by a net, put in a plastic bucket with enough water (5 l), brought from their holding tank, and transferred to the bioassay experimental tanks. Using the same net, prawns were collected from the bucket and placed inside the designated chamber. Each prawn in both chambers was acclimatised inside the bioassay tanks for 30 minutes

where the water temperature is within the range of $28 \pm 1^\circ\text{C}$ and the dissolved oxygen is above 7.0 mg l^{-1} (similar to the HT). Prior to running the test trial, the bioassay tanks were placed and adjusted to the floor level of the wet laboratory. Also, the camera was sited on standby position to be operated by the remote control device. At the beginning of each trial, the camera was turned on (by using a remote control device) immediately after leaving the wet laboratory. By the aim of a TV, behavioural observation was held in the next dry laboratory. Using the remote control device, the camera switched off once the assigned time (30 minutes) is passed over and then water valve turned off. To avoid the possibility of any effect of direction factor or any possible factor, for each tested prawn this procedure was repeated four times by turning around the bioassay tanks direction (two in one direction and other two on the opposite direction). In other words, each behavioural test runs five times 1 as a negative control (plain water, see Control test section 2.2.7) and 4 as actual trials (2 front and 2 back). Each individual behavioural tested prawn was observed, recorded and results saved in a spreadsheet. Prawns were then returned to their holding tanks in the main system.

Immediately, after each single trial, Carapace length of each prawn used in the trial was measured and recorded to minimise stress from handling before trial and then prawns returned to their own tanks in the main system. Then, bioassay tanks were emptied from water, rinsed with clean, fresh and hot water (around 60°C), brushed with a sponge containing a general purpose detergent¹ and then rinsed at least three times with fresh

¹ Active Bactericidal Hand Dishwashing Detergent by Johnson Professional Limited TM, Frimley Green, Camberley, England, UK.

water. This cleaning procedure was applied to avoid any detergent and odours residues which may affect on response behavioural to the following trial. So, these tanks are ready for another trial. This procedure was repeated for each trial and for every individual prawn throughout all the experiments.

2.2.7 Control test

Several trials were conducted in this study as control test. For example, freshly-moulted post-larvae (immature) females of *M. rosenbergii* were used in each trial as a positive control to test their sexual behaviour responses against SM, OC and BC males in order to determine whether or not the moult odour alone from immature female may play any role in sexual attraction. Series of negative controls were also used like plain water, and plastic tubing were conducted prior every testing prawn treatment group. In all control trials, behavioural responses were videotaped, observed, recorded and results were saved in the data spreadsheet.

2.2.8 Sexing prawns

At least five months prior to the experimental trials, *M. rosenbergii* juveniles were sexed and individually housed in the main system. Throughout the experimental trials, prawns used in this study were not only sexually mature but also they were large (more than 30mm CL). Sexing prawns procedure for both males and females were first checked according to their sexual secondary characteristics such as the location of the two sexual gonopores and the presence or absence of the appendix masculina (see section 1.7). Each individual prawn was checked under a dissection microscope for the presence of the appendix masculina. Male morphotypes were categorised according to

their body morphological appearance such as claw size, thickness and coloration into BC, OC and SM (see section 1.6).

In addition, with the aid of a continuous moulting record, it is possible to estimate the females' ovarian maturation stage. Based on a classification discussed by Chang and Shih (1995), ovarian development in female of *M. rosenbergii* can be classified into five stages according to the size and the colour of ovary observed through the carapace. However, instead, females in this study were categorised into three stages of ovarian development, the pre-moult, newly-moulted and inter-moult. Thus, it is possible to categorise female ovarian stages from observations by naked eyes to the ovaries size and colouration.

Prawns were checked for moulting every morning, seven days a week. Tested prawns were selected according to the prawn moulting status (for males and females) and morphotypes (males). Usually, the ovary at the newly-moulted stage is fully mature and orange in colour. The post-moult stage, however, is considered from 1-3 days after female moult. Finally, the inter-moult stage is considered when ovaries have medium size and their moult records fit within 10-16 days after latest moult. If any recently moulted female with full ripe ovaries were found, it was used for a behavioural trial.

To avoid misjudged ovary maturation and to conform that these females are having a full ripe ovary, at the following day after each trial, females which have been used for behaviour test were checked whether they have released their eggs or not. Thus, only

behavioural responses from those females who released their eggs (unfertilised, usually orange colour) within 36 hours after the behavioural test were counted. Whereas, females that did not release eggs within 36 hours after the test were excluded from the experiment and their responses results were ignored.

2.2.9 Urine collection

Several attempts to collect urine from the BC male were made by catheterization (described in Breithaupt *et al.*, 1999, for the lobster, *H. americanus*; Zulandt-Schneider and Moore, 2000, for the crayfish, *P. clarkii*) by using flexible plastic tubing glued to the shell surface surrounding both nephropores. As a part of this study, several attempts were made for *M. rosenbergii* but, unfortunately, these attempts failed. Probably, due to the nephropores position and their shape which make it difficult to fix the catheter tubing around the cuticle opening of the nephropores. Also, the glue had likely blocked the BC nephropores causing unreleased urine, swollen from fluid retention and in most cases the prawns were overstressed and then died within 24 h. Thus, an alternative technique was used which was less stressful to the prawn and certain in collecting urine. Prior to starting applying this technique, BC prawns were anaesthetised for few minutes and then both nephropores (urine opening) and surrounded areas were gently cleaned and dried with disposable cotton tissue. A pair of clean Eppendorfs (1.5ml each) was held against each nephropore. On both sides of their carapace, a gentle pressure (squeeze) were applied and the abdomen was bent down forward meanwhile. Urine was injected directly into Eppendorfs and then each vial was labelled. These steps were repeated with every BC male that was required and fresh collected urine was pooled and used immediately (for fresh urine experiment) or stored in fridge at 4°C or in a in a deep freezer at -70°C.

2.2.10 Anaesthesia

The anaesthesia used in this study is similar to that used with other crustacean species which is employed to all the cases of BC during urine collection. Briefly, in a clean plastic bucket, the anaesthetic solution was prepared by combining 5 drops of clove oil and mixed with 95% alcohol and then poured in 1 L of water (from the rearing tank). Only healthy BC males were chosen individually from their tanks in the main system and placed in the anaesthesia solution. Every BC male was kept in the anaesthetic solution for about 5-7 minutes then picked up, dry out their body surface using soft tissues. After collecting urine, the BC was placed in the recovery bucket containing water from the main system with two aeration air stones. Once fully alert again, they were returned to their tanks.

2.2.11 Characterising urine

A question was raised whether temperature could have an influence on the pheromone quality that may affect on that chemical substance involved in sexual attraction behaviour or not. So, different temperature treatment were conducted on fresh urine collected from the BC males to investigate how the temperature can affect on the key substance that elicits sexual response in the female. Accordingly, fresh urine was collected and exposed to different temperature treatments (4°C, -70°C and 70°C). So, fresh urine was collected in Eppendorfs (micro centrifuge tubes) from the BC males and stored inside a fridge at 4°C for at least one week to be used in cold urine tests. Similarly, fresh urine was collected from the BC males inside the Eppendorfs and then stored in a deep freezer at -70°C to be used in frozen urine tests. Also, fresh urine was collected inside Eppendorfs from the BC males and the Eppendorfs were placed inside a water bath at 70°C for 1 hour to be used in heated urine test. Different volumes (100,

200, 300, 500 and 1000 μl) of each of the above temperature treated BC male urine were tested against newly-moulted females. These urine volumes were injected into the source chamber (A), however, the total volume of water in the system was $\sim 100\text{ l}$ so that at maximum dilution these volumes would have provided concentrations of ~ 1.0 , 2.0, 3.0, 5.0 and $10\mu\text{l l}^{-1}$ for the whole system. Local concentrations of urine, *e.g.* at the entry point from the source chamber into the test chamber, would, however, have exceeded these concentrations. Using the CCVS protocol (see section 2.2.6 page 62), all sexual responses were observed, recorded and results saved in a spreadsheet.

2.3 Part II: Histological work for the antennal gland of *M. rosenbergii*

2.3.1 Antennal gland preparation

All the histological works in this study were carried out according to the routine procedure used at the Histopathology laboratory, Institute of Aquaculture, University of Stirling. These methods modified from Carltons' Histological technique (Drury and Wallington, 1980). However, the antennal glands tissues went through this procedure which follows several steps as demonstrated below.

2.3.2 Tissue fixation

Sample of antennal gland were cut to an appropriate size to allow adequate penetration. Samples were then immersed in at least ten times the individual sample volume of 10% of Neutral Buffered Formalin (NBF) which used as a fixative solution for at least 24 hours.

2.3.3 Cassetting

Plastic cassettes with metal lids were used to carry a single antennal gland each. Each cassette was labelled with a reference number. Those cassettes were then placed in a bowl containing distilled water to be ready for next processing step.

2.3.4 Processing

From the previous step, cassettes along with tissues were then placed into a designated basket which then inserted into a basket carrier, clipped onto tissue processor, which move from stage to stage automatically at a pre-set time intervals as shown in the Table 3 below.

Table 3: Illustrates the materials, their concentrations and the sited time for each steps of processing phase.

Material and Concentration	Time (Minutes)
50% Methylated Spirit	30 min.
80% Methylated Spirit	90 min.
100% Methylated Spirit	90 min.
100% Methylated Spirit	90 min.
100% Methylated Spirit	90 min.
100% Ethanol	1 hour 45 min.
100% Ethanol	90 min.
Chloroform	50 min.
Chloroform	50 min.
Molten Wax	1 hour 45 min.
Molten Wax	90 min.
Molten Wax	90 min.

2.3.5 Blocking out (embedding)

On the next day, cassettes were removed from the tissue processor and placed in the auxiliary wax bath on the histoembedder machine (Histoembedder R134a, Leica Instrument GmbH, Germany). The metal lids were removed from the cassette and appropriate size of base mould (metal) was selected. The base moulds were filled slightly with wax and specimens were gently pick up and positioned into central part of the base mould. Then the base moulds were completely filled covering the specimen to provide an adequate margin of wax around the tissue and to support it during section cutting. The base moulds were then placed on the cold plate upon Histoembedder to solidify the wax block.

2.3.6 Trimming and cutting

The excess wax which might stick around the edges of blocks was removed by scalpel. Using a rotary microtome (Biocut 2035, Leica Instrument GmbH Germany) wax blocks were trimmed to smoothen and to expose the complete tissue surface to be visible to cut. Before sectioning, the trimmed blocks were soaked in distilled water bowl to moistening the tissue prior to cutting for around 30 minutes. Then, blocks were placed facing down on top of cold plate surface (RA lamb, England) for 5–10 minutes. Then blocks were turned facing up. Using the same microtome, wax blocks along with specimen tissue were cut at 5 μm . Individual sections were floated out onto the surface of warmed water in the water bath (Raymond A, Lamb, UK) at 45°C and then mounted on a labelled (identified) microscope glass slides. Slides with specimen tissues were placed side down against the runners on the hotplate (Raymond A, Lamb, UK) which set at 40°C for around one hour to dry them out from attached moist. Then slides were placed into slide racks (25 slides each). To fix the sections onto the slide and to prevent them from floating off during staining, slide racks were transferred to a drying oven (Windsor Incubator, Sand rest, England) at 60°C for overnight before staining.

2.3.7 Staining

Where general visualisation of internal antennal gland structure was required, batches of 25 slides at a time were placed into a slide rack and stained routinely in the Histology laboratory at the Institute of Aquaculture, University of Stirling. The present study routinely staining technique utilised the combines an alum haematoxylin called Mayer's with a counter stain of 1 part 1% Eosin (aq) to 8 parts Putt's eosin. The protocol of this kind of staining is illustrated and described in the Table 4 below.

Table 4: Staining protocol materials and time duration.

Chemical/Solution	Time
Xylene (dewaxing 1)	3 min
Xylene (dewaxing 2)	2 min
Ethanol 1	2 min
Methylated spirit	1 min
Wash in water	30 sec. – 1 min
Haematoxylin	5 min
Wash in water	30 sec. – 1 min
1% Acid alcohol	3 quick dips
Wash in water	30 sec. – 1 min
Scotts tap water substitute	1 min
Wash in water	30 sec. – 1 min
1 part 1% Eosin (aq) to 8 parts Putt's Eosin	5 min
Wash in water	30 sec. – 1 min
Methylated spirit	1 min
Ethanol 2	2 min
Ethanol 3	1 min
Xylene (cleaning)	5 min
Xylene (coverslipping)	

After the final xylene cleaning bath, slides were wiped cleaned with tissue paper and coverslips mounted with Pertex. Slides were allowed to dry in the fume hood for around one hour at room temperature before examination under light microscope.

2.4 Part III: Electron microscopy for the antennal gland of *M. rosenbergii*

2.4.1 The transmission electron microscope (TEM)

Also, some AG samples were prepared to be used under the TEM. Prior to start processing for the TEM, AGs from a designated prawn individuals were surgically removed and then placed individually in glass vials containing 2.5% gluteraldehyde fixative solution and stored at 4°C for at least 2 hours. Specimens then were rinsed in a buffer solution (50% of osmium and 50% of 0.2 M sodium cacodylate) at 4°C and left overnight. Tissues were transferred to 1% of osmium in cacodylate buffer in closed vials inside a fume cupboard and left for 1 hour. Using the rotator, samples were washed three times for 10 minutes each with distilled water to get rid off any residues of the buffer solution (sodium cacodylate) because this buffer solution is incompatible with the uranyl salts in the next step. The AGs were en bloc in a stain of 2% uranyl acetate with 30% of acetone and then kept in a dark container (box) for 1 hour. Series steps of dehydration held in a room temperature through different percentage of acetone as follow and in a different duration as follows:

60% of acetone for 40 mins.

90% of acetone for 40 mins.

100% of acetone for 1 hour.

100% of acetone for 1 hour.

In the fume cupboard the AGs samples were infiltrated with Agar low viscosity resin (ALVR) using the rotator at different percentage of substances as follows:

acetone : ALVR at the ratio of 1:1 for 1 hour.

ALVR at concentration 100% for 1 hour.

ALVR at concentration 100% for 1 hour.

After that, tissues were embedded in either block moulds or Beem capsules. Samples were then polymerised in an oven at 60°C for 24 hours. Specimens were investigated under a TEM (FEI TECNAI SPIRIT G2 BIOTWIN) and the images were printed in special photographic papers.

Chapter 3 Moulting cycle and morphotype mate seeking behaviour

3.1 Introduction

Mating behaviour in crustaceans is closely related to moult cycle, ovarian maturation and a numbers of other factors which may play a complex interaction. Several studies have focused on the interactions between sexual attraction, mating, courtship and moulting stage of crustaceans. Earlier, Templeman (1934, 1936) reported that the best mating time for the *H. americanus* is soon after females' moult. Hartnoll (1969) reported that the copulation is coincident with the female moult and mating occurs shortly after moulting. Similar findings were reported in lobsters, *H. americanus* (McLeese *et al.*, 1977; Cowan and Atema, 1989) and some other crustaceans such as the spider crab, *L. emarginata* (Hinsch, 1968). Similarly, mating of some freshwater prawn species is also controlled by the moult status of the female and the social status of the male morphotype such as in *M. australiense* (Ruello *et al.*, 1973; Lee and Fielder, 1982) and *P. pacuidens* (Kamiguchi, 1972). For *M. rosenbergii*, copulation usually takes place shortly after the female moult (Ling 1969; Chow *et al.*, 1982; Thomas, 1998).

Since this kind of reproductive behaviour is related to the moult status and ovary maturation state of females and to the morphotype of the males, it is suggested that this behaviour is controlled by different types of communication means, where chemical cues play a considerable role. It is clear from the literature cited that chemically mediated pre-copulatory attraction is a key component in the reproductive behaviour of many species of aquatic crustacean and that attractive odours (pheromones) may be released by males and/or females according to species. Moreover, it is clear that attractiveness may be closely associated with the moult stage and/or the reproductive state (*e.g.* gravid/non-gravid). From these mentioned studies it seems that reproductive

behaviour in *M. rosenbergii* is also likely to be subject to the same controlling influence. However, details in the effect of moult status and male morphotypes on sexual attracting behaviour are demanded.

3.1.1 Effect of female's moult cycle on sexual attraction behaviour

Several studies on the sexual attraction behaviour of female crustacean showed the involvement of moult cycle and ovarian maturation sexual attraction behaviour using chemical cues. The existence of the correlation between the female's moulting, ovarian maturation status and sexual attraction of receptive males during sexual attraction behaviour is also reported in several crustacean species such as the spider crab, *L. emarginata* (Hinsch, 1968) and the lobster, *H. americanus* (McLeese *et al.*, 1977), who found a strong correlation between the female's moulting and ovarian maturation status with sexual attraction of receptive males where chemical cues, possibly a sex pheromone, play a major role during sexual attraction behaviour. Earlier, Atema and Engstrom (1971) demonstrated that male of lobsters, *H. americanus* reject females at inter-moult probably due to the lack of the proper pheromone. Atema and Cowan (1986) reported that males showed a typical response to the urine and moult body odour from female lobsters, *H. americanus* that had recently moulted. Later, Cowan and Atema (1989) found that mating is associated with the time of female's moulting cycle, where sexual behaviour commenced just prior to a mature female moult.

A number of studies have been conducted to test male behaviour with respect to different moulting stages of female crustaceans. Ryan (1966) studied the sexual attractiveness of the edible crab, *P. sanguinolentus*, and observed that males displayed a

positive response to the presence of pre-moult females. Evidence of sexual attraction was observed when water was siphoned from a tank containing females into a tank containing males (Ryan, 1966). He attributed these behavioural responses to the existence of a pheromone in the water from the females' tank. Thus, they indicated that a sexual attractive substance was emitted which functioned as a releasing stimulus evoking the search phase of the males' behaviour.

Ryan's hypothesis was confirmed later by Eales (1974) who showed that the pre-moult female shore crab, *C. maenas*, released a pheromone into the water, via the urine, which attracted the male. Hartnoll and Smith (1979) noticed that mating occurs only between hard shelled male edible crabs *C. pagurus* and recently moulted females. Bamber and Naylor (1996a) found that males of *C. maenas* showed a very low level, or absence, of response to the inter-moult females compared to the pronounced responses to the pre-moult females. Later, Bamber and Naylor (1997) concluded that urine from *C. maenas* pre-moult females is a source of a putative sex pheromone which attracts males. Hardege *et al.* (2002) demonstrated that male mate selection in *C. maenas* is based largely on the detection of female odour compounds. When water was collected from tanks containing the pre-copula females, positive responses were observed from sexually mature males, whereas water collected from tanks containing inter-moult females failed to induce a sexual response to those males. Gleeson (1980) observed that the only copulation of the blue crab, *C. sapidus* female occurred immediately after the puberty moult (mating moult), as a typical soft-shell mating. Thus, he suggested that a pheromone was released in the urine of puberty-moult females. Briones-Fourezan and Lozano-Álvarez (2005) studied the seasonal variations in chemical responses to

conspecific scents in the spotted spiny lobster, *Panulirus guttatus*. These authors also noticed that when both male and female spotted spiny lobsters are at high reproductive season, males responded significantly to females.

Moreover, Jones and Hartnoll (1997) ran chemical communication experiments to test the sexual attractiveness behaviour of post-moult stage females of spider crabs, *I. dorsettensis* to the males. Results of their studies showed that the post-moult females with either eggs near hatching, or which have recently hatched their eggs, are attractive to males and mate readily. They concluded that there is a correlation between ripe ovaries and attractiveness.

Using conditioned water on snapping shrimp, *A. heterochaelis*, Hughes (1996) tested the influence of moulting status (post and pre-moult) of females on the attractiveness behavioural responses of males. She found that when females ($n = 9$) are not carrying eggs, none of the males ($n = 12$) were able to differentiate between the sea water (control, with no shrimp) and the conditioned water carrying chemical signals of both the post and the pre-moult females. Similarly, she found that none of the females in these statuses (post- and pre-moult) responded to the males. When females carrying eggs (pre and post-moult) were used in the test, Hughes (1996) found neither males ($n = 5$) nor females ($n = 11$) responded to the opposite sex. She concluded that snapping shrimp do not respond to chemical signals alone and other communicational cues have to be involved. Another study conducted using a Y-maze experiment to test chemical communication in *A. angulatus*, Mathews (2003) found males were attracted to water treated by exposure to pre-moult females but were not attracted to water treated by

exposure to the inter-moult females. Furthermore, in mate choice experiments, she found significantly more males paired with the pre-moult than with the post-moult females.

For copepods, Watras and Hanery (1980) suggested that the female *Diaptomus leptopus* must be gravid at the time of copulation for a viable clutch to be produced. Also, in mating experiments, Chow-Fraser and Maly (1988) found males of three freshwater calanoid copepods, *Epischura lacustris*, *D. minutus* and *D. oregonensis*, are significantly more attractive to gravid females than non-gravid females. Later, in behavioural experiments, Leeuwan and Maly (1991) found significant alterations in swimming behaviour of *D. leptopus* only in the gravid females. They suggested that gravid females may produce chemicals that are different from those of non-gravid females. They concluded that male *D. leptopus* copepods are able to distinguish the reproductive state of the female and probably do use pheromones to enhance rates of copulation.

Thomas *et al.* (1998) studied the influence of time left for male moult as a factor on the mating process with female amphipods, *Gammarus aequicauda*. They found that male about to moult were significantly showing less interest to mate with females compared with males who were far from moulting. They referred such behavioural action to the fact that male close to moult are less active which then encounter fewer females than the type of males. Also, as the males' moult approaches, in order to secure mating these males might accept females with a lower critical value because males close to their moult have less time available for mate acquire. It is highly possible that the above

reasons can be applied to the freshwater in this study in which the newly moulted BC male prawns were least attractive to the receptive females compared to those pre- and inter-moult BC males who are away from moulting.

Kamiguchi (1972) found low levels of sexual response when water collected from a container containing inter-moult or pre-moult females of the freshwater prawn *P. paucidens* was used to test 50 sexually mature males. However, when water collected from a container containing soft-shelled (newly-moulted) females was used, males showed high level of sexual response (92%, 46 out of 50 males). Meanwhile, no sexual response was observed in *P. paucidens* males that were placed together with the inter-moult (stage C) or pre-moult (stage D) females. He concluded that the soft shell of females might be another important factor for eliciting the mating behaviour response.

3.1.2 Effect of male's moult cycle on sexual attraction behaviour

In contrast to the number of studies conducted on female attractiveness, the effect of male's moult status on sexual attraction of receptive females seems to be limited. Atema and Cowan (1986) conducted an experiment testing the sexual behavioural response of female and male lobsters, *H. americanus*, to urine and body odour collected from males. They observed that the female response was weak to males at the inter-moult stage. They, therefore, suggested that moult body odours and inter-moult urine contained sex-specific substances that might be used in lobster, *H. americanus*, courtship. For spider crabs, *I. dorsettensis*, Jones and Hartnoll (1997) found that recently post-moulted females are not attractive to the inter-moult males.

In a comparable experiment studying copulation behaviour of 50 sexually mature males of the freshwater prawn *P. paucidens* in different moult stages (A, B, C, D₁ and D₂₋₃), Kamiguchi (1972) found males at stages C and D₁ had a better chance (90% and 80%, respectively) to copulate with newly-moulted females than the other moult stages A, B, and D₂₋₃ (0%, 30%, and 30%, respectively).

Another study, Parnes *et al.* (2006) showed that male shrimp *Litopenaeus vannamei* had went through reproductive cycles that are strictly associated with their moult cycles. They noticed that spermatophores are periodically disappeared from the terminal ampoules of males during the 24 h pre-moult. Meanwhile, they noticed a new pair of white spermatophores reached the ampoules on the night of ecdysis following the moult. The nature of the association between moult cycles and reproduction in *M. rosenbergii* is not known. Thus, more work emphasis in finding the influence of moult status and sex attraction is needed in order to understand the nature of such behaviour dynamic.

3.1.3 Effect of male's morphotypes on sexual attraction behaviour

Adult crustacean individuals could have some restriction in choosing their partner. Several studies indicated that females are capable of exerting preferences for certain males by exhibiting resistance to some but not all male types. For instance, female crayfish, *O. rusticus* resisted mating attempts from small males more efficiently than those from large males (Snedden, 1990). Jivoff and Hines (1998) reported that large male blue crab, *C. sapidus* had advantages in displacing smaller males from females and in preventing their own displacement. Female isopods, *Idotea baltica*, resist

approaching males more intensively and successfully in less size-dimorphic populations (Jormalainen *et al.*, 2000).

Male rock shrimp, *R. typus*, undergo strong morphological changes during ontogeny: most males reach sexual maturity in the female-like typus stage, and during growth they pass through several intermedium moult stages before reaching the final stage, termed robustus (Correa *et al.*, 2003). According to shrimp's dominance hierarchy, size and morphology, Correa *et al.*, (2003) identified three male morphotypes of rock shrimp, in which the robustus is the most dominant and bigger in size followed by the intermedium and then the sub-ordinate typus stage. Díaz and Thiel (2003) conducted an experiment to test female rock shrimp, preference of choosing males. In their experiment, inside a large tank 140 x 60 x 30 cm (L, W and D, respectively) with standing seawater, 11 replicate females were used to choose between two different males morphotypes (typus and robustus). They found that 10 out of 11 females had preference for dominant males (robustus) during mating more than the sub-ordinate males (typus) and females remained in the vicinity of the robustus.

Later, using a Y-maze design, Díaz and Thiel (2004) conducted another experiment in which examining whether receptive females and the most dominant male, robustus, of *R. typus* utilise chemical cues, visual cues, or both to locate and assess a potential mating partner. They observed that females approached the robustus males significantly more often than the typus males. Also, they demonstrated that receptive females do not use visual cues to select robustus males, but robustus males use visual cues to find

receptive females. They concluded that females and males use different communicational signals during mate searching and assessment.

Thus, it is necessary to get more understanding view of the interactions behaviour between individuals within a population for reproductive activities and agonistic behaviour of *M. rosenbergii*, especially those are related to chemical communication. The effect of moult stage of females and male morphotypes in mating preference is the main focus in this part of this study. So, testing only chemical cues and restricting both visual and tactile communication between individuals during reproductive period, may give a better understanding of the complex interaction between males and females using only chemical communication cues. For this purpose, as demonstrated later in this chapter, the first experiment was constructed into three trials each in which has sub-aim.

3.2 Main objective

The main objective of the experiments described in this chapter was to investigate the pattern of chemical communication between males and females *M. rosenbergii*. Also, to establish whether there is any effect of moulting stage of both males and females, and of male morphotype on sexual attraction in this species. These main objectives are described as follows:

- 1- To characterise the effect of the moulting status of sexually mature females at different moult stages on the sexual attraction behaviour of the BC male *M. rosenbergii*.

2- To characterise the effect of the moulting status of BC males at different moult stages on the sexual attraction behaviour of the newly-moulted females *M. rosenbergii*.

3- To characterise the effect of the male morphotype on the sexual attraction behaviour of the newly-moulted females *M. rosenbergii*.

At the end of these three trials, it was expected to have a better understanding of the role of chemical communication cues, if any, between individuals *M. rosenbergii* with respect to reproductive behaviour. Descriptions of the protocols used for each trial are provided in the next sections and the results are given in the following sections.

3.3 Materials and methods

The bioassay experimental tanks (see section 2.2.4 and Figure 9) were constructed to study the effect of chemical cues on mating behaviour between male and female *M. rosenbergii* at different moulting stages and with different male morphotypes. However, this experiment consists of three trials which attempt to answer certain questions related to the chemical communication between males and females.

3.3.1 Trial 1: The effect of female's moult cycle on sexual attraction behaviour

The bioassay tanks (see section 2.2.4 and Figure 9) were prepared to run the first trial of this experiment. The main objective of this trial was to investigate the effect of the three moult stages of female *M. rosenbergii* (pre-moult, newly-moulted and inter-moult, see section 1.7 page 19) on sexual attraction behaviour of BC males. Twelve individual

females from each of the above moult stages were used. Each single female was run five times, 1 as a control (blank freshwater) and 4 with tanks in two orientations, two forward and two reverse, in order to avoid additional environmental influences. For each replicate, a single female was placed in the source chamber to be tested with a single BC male which was placed in the test chamber. Prior to running the four runs, both the male and the female were placed in their designated chambers for 30 minutes for acclimation. Each run lasted 30 minutes starting immediately after turning the water valve on. Behavioural attraction of the BC males was video recorded and the behavioural response times were recorded and then analysed (see section 2.2.6 page 62).

3.3.2 Trial 2: The effect of male's moult cycle on sexual attraction behaviour

The main objective of this trial is to investigate the effect of the moult stages of BC male *M. rosenbergii* (pre-moult, newly-moulted and inter-moult) on sexual attraction behaviour of the newly-moulted females. In order to avoid additional environmental influences, each single male was run four times with tanks used in two positions, two forward and two reverse. For each replicate, a single male was placed in the source chamber to be tested, with a single newly-moulted female placed in the test chamber. Prior to running the four runs, both the male and the female were placed in their designated chambers for 30 minutes for acclimatisation. Each run lasted 30 minutes starting immediately from the time of turning the water valve on. Behavioural attractions of the newly-moulted females were video recorded and the behavioural responses time of BC male were recorded and then analysed.

3.3.3 Trial 3: Effect of male's morphotypes on sexual attraction behaviour of females.

The main objective of this trial was to investigate the effect of three morphotypes of male *M. rosenbergii* (SM, OC and BC) on sexual attraction behaviour of the newly-moulted females. From each of the above morphotypes, 12 individual males were used, with each individual male run four times, two forward and two reverse in order to avoid any environmental factors that might influence the results. For each replicate, a single newly-moulted female was placed in the source chamber to be tested with males from each of the above male morphotypes placed in the test chamber. Prior to running the four runs, experimental prawns were placed in their designated chambers for 30 minutes for acclimation. Each individual run lasted 30 minutes starting immediately after turning the water valve on. Behavioural attractions of male morphotypes were video recorded and the behavioural responses time were recorded and then analysed.

3.4 Statistical testing

Results were analysed using a repeated measures general linear model (GLM). Although samples did not all show homogeneity of variance or normal distributions, and were not responsive to transformation (Log10, reciprocal and square root), it is recognised that ANOVA models are relatively robust to failures to meet assumptions of normality and homogeneity of variance (Jackson and Brashers, 1994) particularly when groups are of equal sample size. Hence these tests should nevertheless provide a good guide to the main effects acting upon behavioural responses, although larger numbers of samples might allow more robust tests in the future. It is also worth noting that the confidence intervals on graphs are for the univariate statistics and therefore will not

accurately reflect the results of the repeated measures GLM in terms of apparent significant differences between samples.

3.5 Results

The sexual responses of *M. rosenbergii* individuals within the three trials are analysed below. Statistical analysis indicates different responses between the two genders according to their moulting stages and depending on male morphotype status.

3.5.1 Trial 1: The effect of female's moult cycle on sexual attraction behaviour.

A Dunnett's test (Table 5) indicated that all females in different moulting stages exposed to BC males water responded significantly more quickly ($p=0.000019$) than those exposed to freshwater controls and this can be seen clearly in Figure 10.

Table 5: Dunnett's test indicating that all runs using females of any moult stage with BC exposure are significantly different from runs using water only (control runs), $n = 12$. Bold indicates $p<0.05$.

Run	Control
Control	29.750
Forward 1	0.000019
Reverse 1	0.000019
Forward 2	0.000019
Reverse 2	0.000019

Table 6: The mean and the standard deviation (SD) of sexual response time (minutes) of the BC males to different moulting stages of females *M. rosenbergii*.

Time (minutes)	Control	Pre-moult	Newly-moulted	Inter-moult
Mean \pm SD	29.75 \pm 0.67	20.56 \pm 7.30	8.48 \pm 5.95	25.02 \pm 5.98

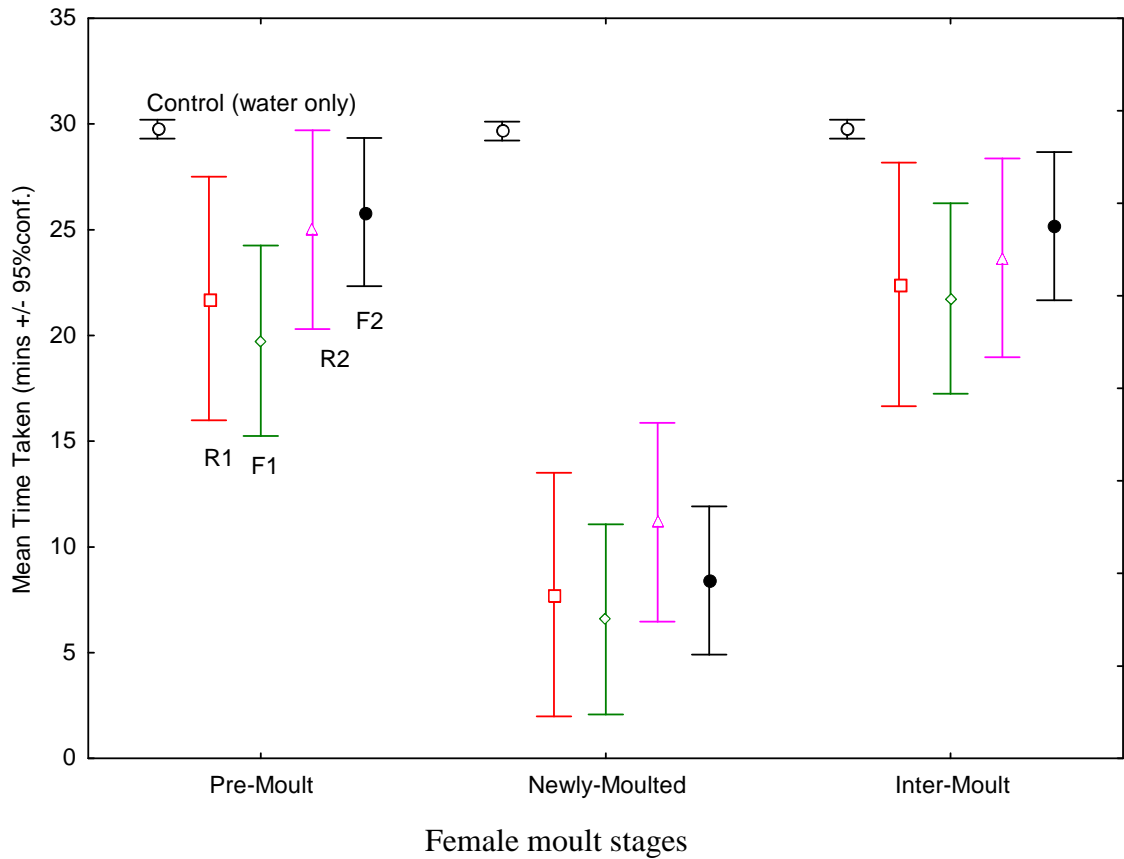


Figure 10: Graph of mean responses of different moult stage of females to BC males showing results for control (water only) and first and second forward and reverse test chamber orientation runs. F1 = first forward run; R1 = first reverse run; F2 = second forward run; R2= second reverse run.

A GLM analysis (Table 7) shows that female moult stage had a significant effect response on BC males (Figure 10). A Tukey HSD *post-hoc* analysis (Table 7) indicates that pre-moult and inter-moult females responded significantly more slowly than new-moult females. Also the experimental run for female moult stage was significant (Table 8 and Figure 12).

Table 7: Repeated measures GLM for effects of different moult stage of females on time taken to reach target by BC males. Run = experimental run; Orient = chamber orientation; Stage = moult stage of male, n = 12. Bold indicates $p < 0.05$.

Source of Variation	SS ¹	DF ²	MS	F	P
Intercept	48070.56	1	48070.56	470.8050	<0.000001
Stage	6903.79	2	3451.90	33.8080	<0.000001
Error	3369.40	33	102.10		
RUN	370.56	1	370.56	4.7575	0.036401
RUN*Stage	38.79	2	19.40	0.2490	0.781021
Error	2570.40	33	77.89		
ORIENT	18.06	1	18.06	0.4644	0.500338
ORIENT*Stage	34.13	2	17.06	0.4387	0.648598
Error	1283.56	33	38.90		
RUN*ORIENT	11.67	1	11.67	0.3292	0.569998
RUN*ORIENT*Stage	34.01	2	17.01	0.4797	0.623244
Error	1170.06	33	35.46		

¹ SS: Sum of Square

² DF: Degree of Freedom

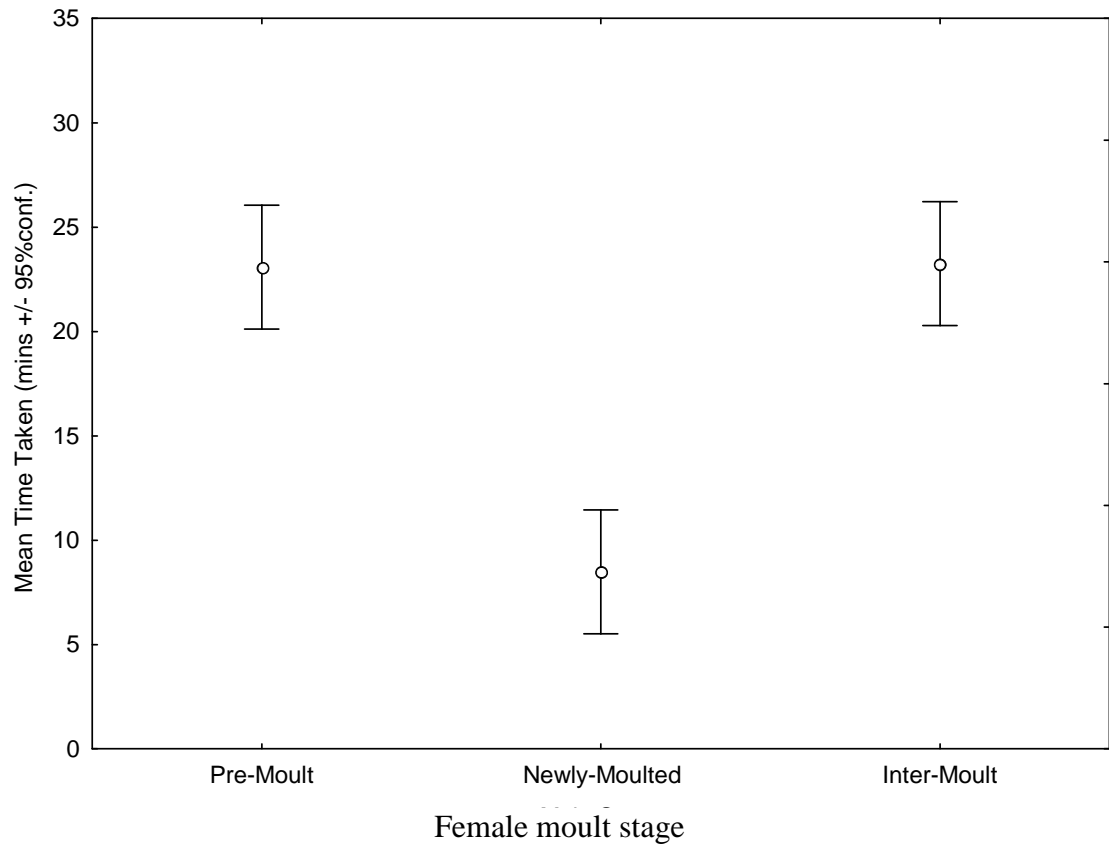


Figure 11: Graph of mean responses time of different moult stage females to BC males.

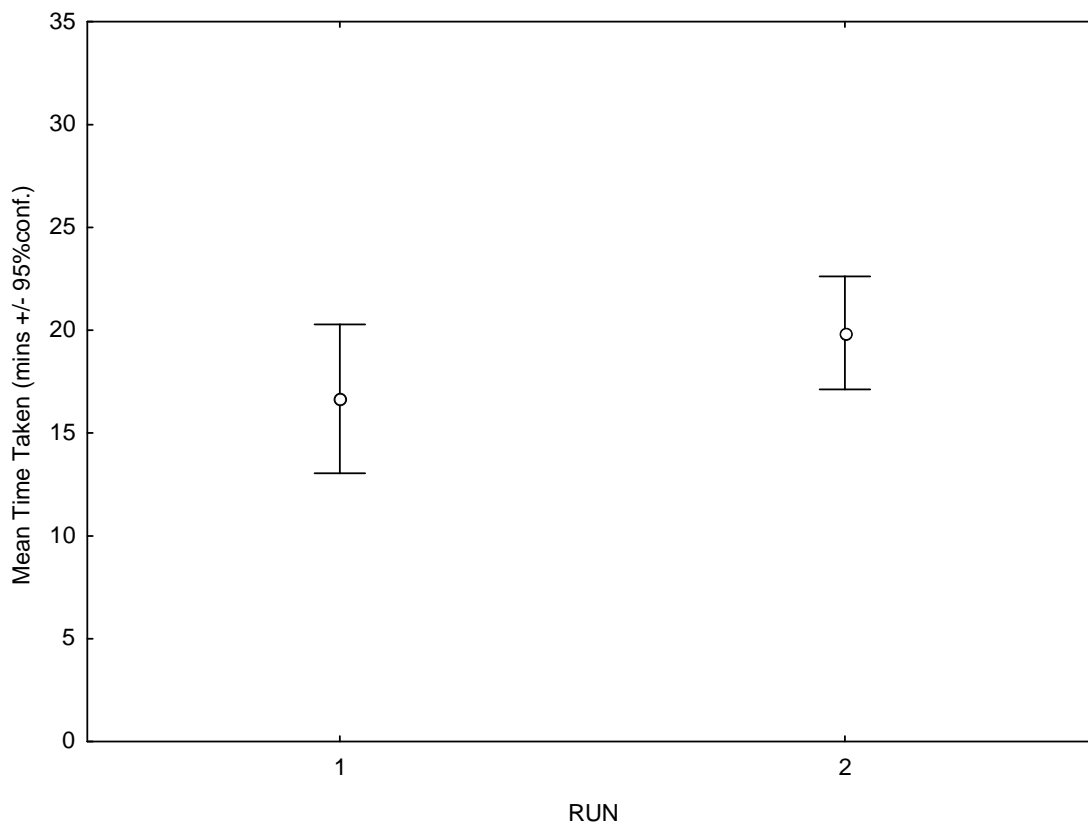


Figure 12: Graph of mean response time of different moult stage females to BC males in two experimental runs.

Table 8: Tukey HSD *post-hoc* test for effects of moult stage of females on time taken to reach target by BC males exposed to different moult stages females, $n = 12$. Bold indicates $p < 0.05$.

Stage	Pre-moult	Newly-moulted	Inter-moult
Pre-moult			
Newly-moulted	0.000126		
Inter-moult	0.996497	0.000126	

3.5.2 Trial 2: The effect of male's moult stage on the sexual attraction behaviour response of newly-moulted females.

A Dunnett's test (Table 9) indicated that all newly-moulted females exposed to male BC (at different moulting stages) water responded significantly more quickly ($p=0.000019$) than those exposed to freshwater controls and this can be seen clearly in Figure 13.

Table 9: A Dunnett's test indicating that all runs using males of any moult stage with newly-moulted female exposure are significantly different from runs using water only (Control runs), $n = 12$. Bold indicates $p < 0.05$.

Run	Control
Control	29.722
Forward 1	0.000019
Reverse 1	0.000019
Forward 2	0.000019
Reverse 2	0.000019

Table 10: The mean and the standard deviation (SD) of sexual response time (minutes) of newly-moulted females to different moulting stages of BC males *M. rosenbergii*.

Time (minutes)	Control	Pre-moult	Newly-moulted	Inter-moult
Mean \pm SD	29.72 \pm 0.67	12.04 \pm 4.75	22.67 \pm 6.29	9.19 \pm 3.99

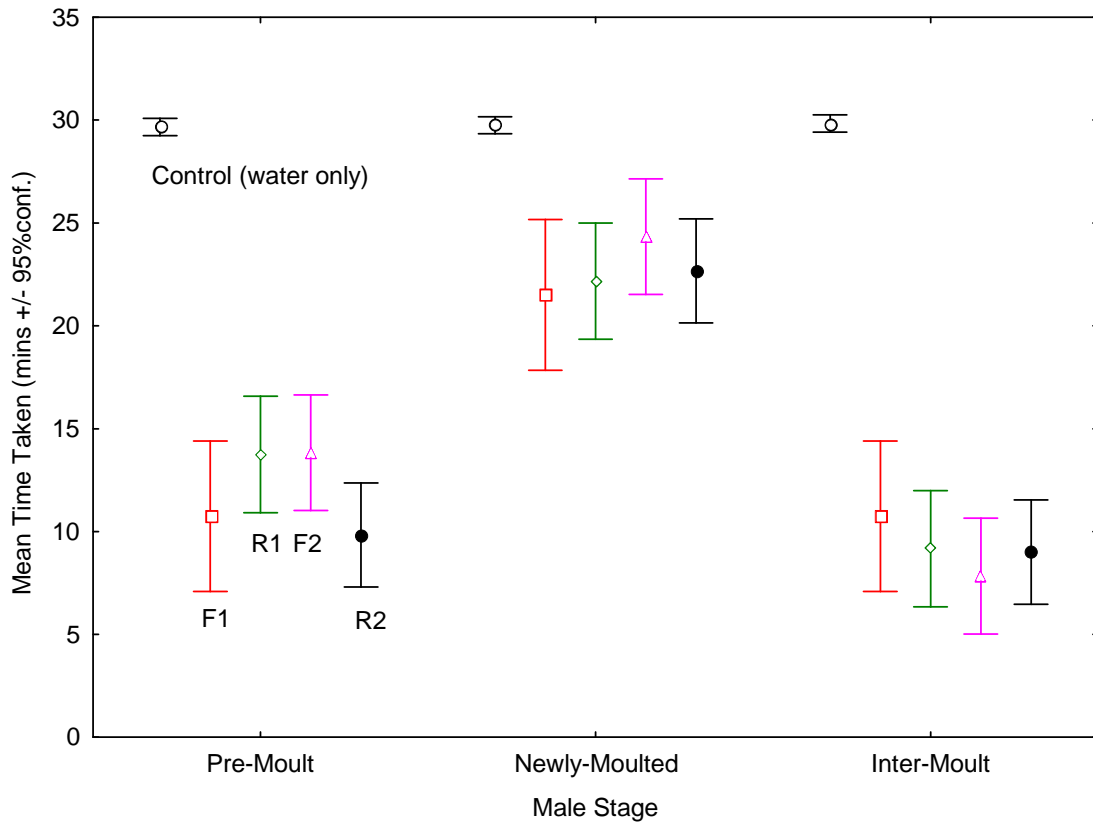


Figure 13: Graph of mean responses time of different moult stage BC males to newly moulting females showing results for control (water only) and first and second forward and reverse test chamber orientation runs. F1 = first forward run; R1 = first reverse run; F2 = second forward run; R2= second reverse run.

A GLM analysis (Table 11) shows that BC male moult stages had a significant effect on BC males to newly-moulted female response (Figure 14) and there was a significant interaction between the run number, test chamber orientation and moult stage of females. A Tukey HSD *post-hoc* analysis (Table 12) indicates that pre-moult and inter-moult BC males responded significantly faster than new-moult BC males.

Table 11: Repeated measures GLM for different moult stage BC males to newly moulting females on time taken to reach target by females of different moult stages exposed to BC males. Run = experimental run; Orient = chamber orientation; Stage = moult stage of female, $n = 12$. Bold indicates $p < 0.05$.

Source of Variation	SS	DF	MS	F	P
Intercept	30829.51	1	30829.51	622.1914	<0.000001
Stage	4843.60	2	2421.80	48.8760	<0.000001
Error	1635.15	33	49.55		
RUN	0.34	1	0.34	0.0247	0.876033
RUN*Stage	63.60	2	31.80	2.3098	0.115123
Error	454.31	33	13.77		
ORIENT	5.84	1	5.84	0.3075	0.582948
ORIENT*Stage	0.68	2	0.34	0.0179	0.982252
Error	626.73	33	18.99		
RUN*ORIENT	43.34	1	43.34	2.0513	0.161481
RUN*ORIENT*Stage	142.68	2	71.34	3.3766	0.046330
Error	697.23	33	21.13		

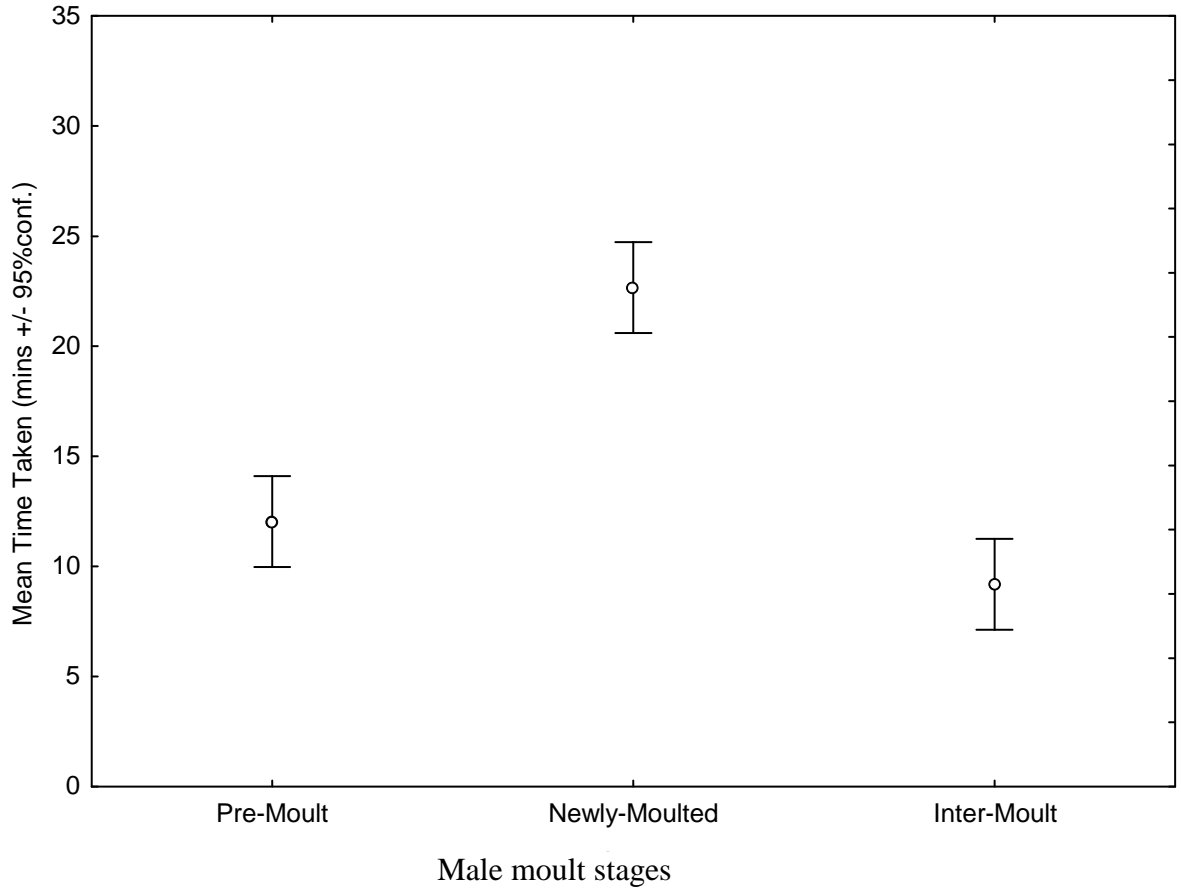


Figure 14: Graph of mean responses of newly-moulted females to different moult stage BC males.

Table 12: Tukey HSD *post-hoc* test for effects of moult stage on time taken to reach target by newly-moulted females exposed to different moulting stages BC males, $n = 12$. Bold indicates $p < 0.05$.

Stage	Pre-moult	Newly-moulted	Inter-moult
Pre-moult			
Newly-moulted	0.000126		
Inter-moult	0.131561	0.000126	

3.5.3 Trial 3: To test the effect of male morphotypes on sexual attraction behaviour of females.

A Dunnett's test (Table 13) indicated that all newly-moulted females exposed to different male morphotypes responded significantly more quickly ($p=0.000019$) than those exposed to freshwater controls and this can be seen clearly in Figure 15.

Table 13: Dunnett's test indicating that all runs using females with all male morphotypes exposure are significantly different from runs using water only (control runs), $n = 12$. Bold indicates $p < 0.05$.

Run	Control
Control	29.78
Forward 1	0.000019
Reverse 1	0.000019
Forward 2	0.000019
Reverse 2	0.000019

Table 14: The mean and the standard deviation (SD) of sexual response time (minutes) of newly-moulted females to different male morphotypes *M. rosenbergii*.

Time (minutes)	Control	SM ¹	OC ²	BC ³
Mean ± SD	29.77 ± 0.72	16.25 ± 5.17	22.67 ± 9.11	8.38 ± 4.27

¹ SM: Small Male

² OC: Orange Claw

³ BC: Blue Claw

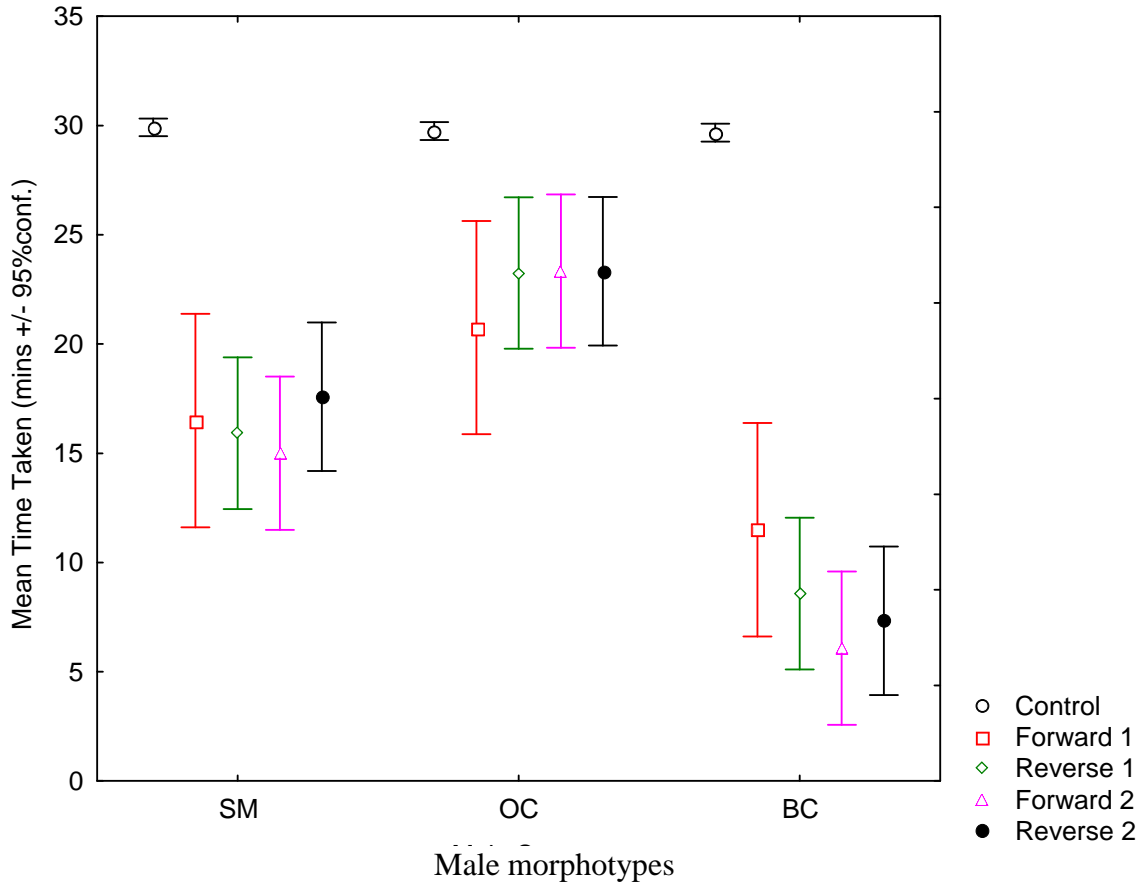


Figure 15: Graph of mean responses time of newly-moulted females to different male morphotypes showing results for control (water only) and first and second forward and reverse test chamber orientation runs.

A GLM analysis (Table 15) shows that male morphotype had a significant effect on newly-moulted female response (Figure 16) and that the interaction between run and test chamber orientation was also significant. A Tukey HSD *post-hoc* analysis indicates that BC males responded significantly faster than SM and OC males and those SM males responded significantly faster than OC males (Table 16).

Table 15: Repeated measures GLM for effects of male morphotype on time taken to reach target by newly-moulted females exposed to different male morphotypes. Run = experimental run; Orient = chamber orientation; Stage = moult stage of male, $n = 12$. Bold indicates $p < 0.05$.

Source of Variation	SS	DF	MS	F	p
Intercept	35784.03	1	35784.03	329.0791	< 0.000001
Stage	4919.06	2	2459.53	22.6184	0.000001
Error	3588.42	33	108.74		
RUN	14.69	1	14.69	0.9068	0.347885
RUN*Stage	140.06	2	70.03	4.3215	0.021530
Error	534.75	33	16.20		
ORIENT	8.03	1	8.03	0.5090	0.480567
ORIENT*Stage	31.06	2	15.53	0.9846	0.384286
Error	520.42	33	15.77		
RUN*ORIENT	23.36	1	23.36	0.7161	0.403528
RUN*ORIENT*Stage	77.56	2	38.78	1.1886	0.317342
Error	1076.58	33	32.62		

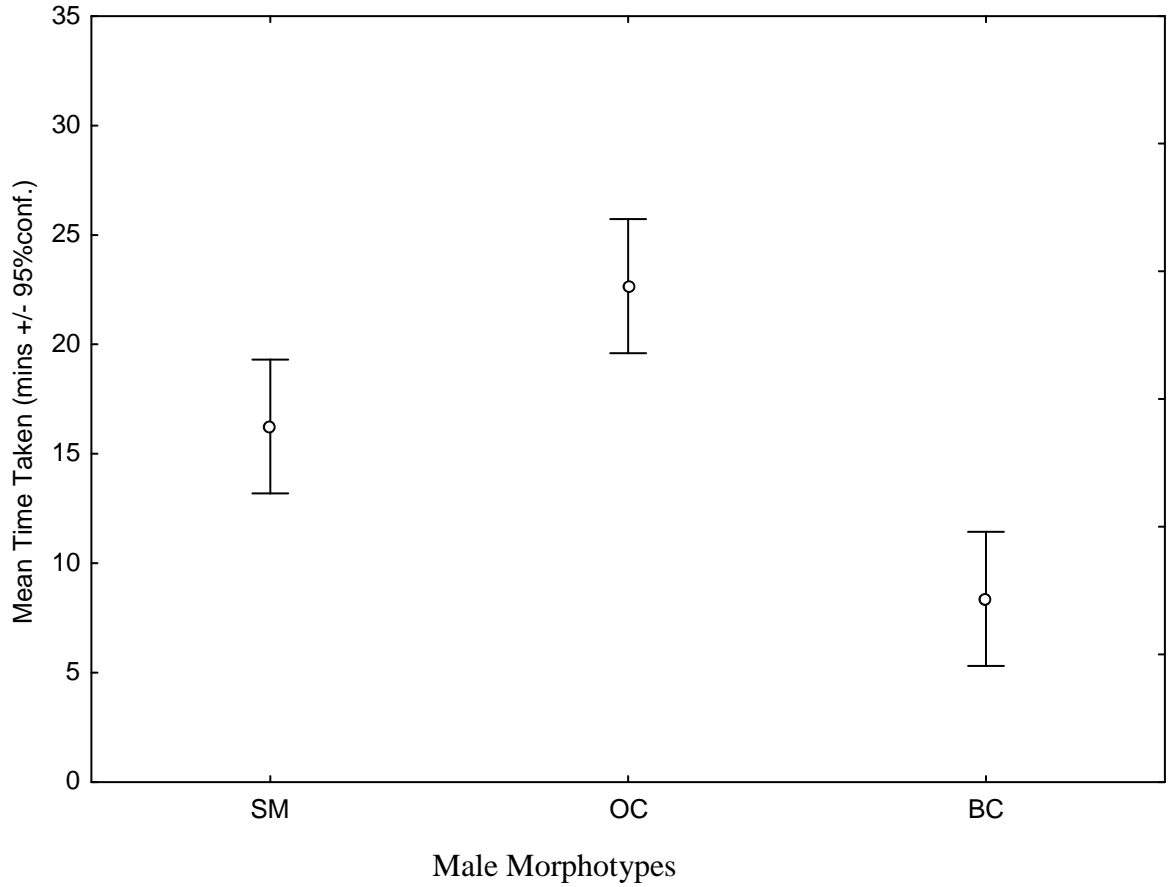


Figure 16: Graph of mean responses time of newly-moulted females to males of different morphotypes

Table 16: Tukey HSD *post-hoc* test for effects of morphotype on time taken to reach target by newly-moulted females exposed to different male morphotypes, $n = 12$. Bold indicates $p < 0.05$.

Stage	SM	OC	BC
SM			
OC	0.013381		
BC	0.002297	0.000127	

3.6 Discussion

Results obtained in this study confirm the relationship between sexual attraction and moulting stage of *M. rosenbergii*. Results in this study indicate that the moult stages of both adult male and female prawns *M. rosenbergii* have a key role in determining the opponent response to water-borne cues. The physiology of *M. rosenbergii* changes dramatically during the moult cycle, which in turn may change both the chemical content and concentrations of the chemical cues released into the water. This can be seen through the attraction behavioural responses. Findings in this study are similar to these found previously for several other several crustacean species such as *H. americanus* (Templeman, 1934, 1936; McLeese *et al.*, 1977; Cowan and Atema, 1989), some Brachyura, (Hartnoll, 1969), the spider crab, *L. emarginata* (Hinsch, 1968), freshwater prawns such as *M. australiense* (Ruello *et al.*, 1973; Lee and Fielder, 1982) and shrimp *P. paucidens* (Kamiguchi, 1972). The findings in this study are consistent with studies conducted previously in the *M. rosenbergii* by Ling (1969), Chow *et al.* (1982) and Thomas (1998). Thus, it can be suggested that the sex attraction behaviour in *M. rosenbergii* is likely to be controlled by chemical cues (pheromones) released by males, the females or both, the release these being strongly associated with the moult stage.

In the experiments conducted there were differences between trial runs, with the second set of runs for each prawn showing responses that were significantly slower than the first. This may be due either to tiring following extensive movement in response to earlier cues or due to acclimation to cues received causing reduced sensitivity (Tinbergen, 1951). There were also interactions between experimental run and moult

stage that may reflect stamina or other differences between different moult stages which change performance following extended exercise.

3.6.1 The effect of female's moult cycle on sexual attraction behaviour.

Results from this experiment provide clear evidence of correlation between the intensity of sexual attraction of the male to females and the ovarian maturation of females *M. rosenbergii*. Blue claw males showed varying degrees of positive response to all tested female prawns relative to the female's moult stages. This verifies not only the existence of chemical cues in the water released from females but also that these chemical cues are varying in their content. Thus, the level of attraction of BC males to receptive female prawns depends strongly on the female's ovarian maturation level. This can be seen clearly in this experiment where the BC males took the least time to move towards the newly-moulted female *M. rosenbergii* compared to the pre and inter-moult females. This incident intimates that newly-moulted female *M. rosenbergii* release distinct pheromone compound that attracts the BC males more than the other females' moulting stages. This is more in line with expected newly-moulted female behaviour and may also reflect a behaviour tending towards being sought rather than seeking during this phase of the moult cycle. Clearly this could reflect the reduced mobility / energy reserves that might be expected following the moult or the behaviours that tend to keep them in shelter at this potentially vulnerable stage (Ra'anan, 1983).

Similar behavioural responses to these displayed by newly-moulted female *M. rosenbergii*, has also been reported in other crustaceans such as edible crab *P. sanguinolentus* (Ryan, 1966), shore crab, *C. maenas* (Eales, 1974) and blue crab, *C.*

sapidus (Gleeson, 1980). Results in this trial parallel to those of Ryan (1966), who suggested the existence of pheromone in water obtained from holding tanks containing pre-moult females of the edible crab *P. sanguinolentus*. The findings of this trial are also consistent with the findings of Atema and Cowan (1986) who reported that male lobsters, *H. americanus* showed positive response behaviour to recently moulted females and they concluded that mating was associated with the timing of the female's moulting cycle. The same relationship between moulting and ovarian ripeness that exists in lobster also exists with the freshwater prawn *M. rosenbergii*. Positive behavioural responses of males with respect to gravid females are also observed in several copepods species such as *D. leptopus* (Watras and Hanery, 1980; Leeuwan and Maly, 1991), *E. lacustris*, *D. minutus*, *D. oregonensis* (Chow-Fraser and Maly, 1988) and the hermit crab, *Pagurus filholi* (Goshima *et al.*, 1998).

In contrast, it is observed that the attraction behaviour responses of the BC males *M. rosenbergii* were fastest toward the newly-moulted female prawns and the slowest response were observed toward both pre- and inter-moult females. This slow response is most likely to be related to incomplete ovarian maturation of these females at these particular moult stages. Another incident for other freshwater prawn species was previously reported. Kamiguchi (1972) conducted an experiment of sexual attraction behaviour in *P. paucidens* using water collected from containers containing different moulting stages of females, the findings of the present study were very much analogous to his results. Similar findings have been reported in some other aquatic crustacean species where the dominant males were not showing interest to the inter- and pre-moult females such as lobsters, *H. americanus* (Atema and Engstrom, 1971); the edible crab,

C. pagurus (Hartnoll and Smith, 1979); shore crabs, *C. maenas* (Bamber and Naylor, 1996a), the blue crab, *C. sapidus* (Jivoff and Hines, 1998) and snapping shrimp, *A. angulatus* (Mathews, 2003)

There is more than one possible reason for BC males showing a slow response to these particular females. Females at pre- and inter-moult stages may perhaps lack the earlier stage sexual pheromone. Alternatively, females at these moult stages may release pheromones inhibiting mating behaviour. Thus, one or more of the above reasons may be involved in sexual attraction behaviour between adult female and male prawns but further research is required to draw a more definite conclusion.

Dunham (1978, 1988) pointed out that chemical cues released from a female which elicit male responses do not necessarily function as sex pheromones. He attributes the male's response to female chemical cues as cannibalistic predators toward potential prey. However, observations in this study about *M. rosenbergii* suggest that BC males responding to chemical cues from a female as signals from potential mates because BC males were significantly attracted to chemical cues released from newly-moulted females more than those released from pre- and inter-moult females. This suggests that chemical cues were probably eliciting mate-searching behaviour rather than cannibalistic foraging behaviour.

As noted from this study, sexual attraction is strongly related to ovarian maturation rather than to moulting, where newly-moulted females have fully ripe ovaries and inter-

and pre-moulted females have un-ripe ovaries. It is suggested that the ovarian maturation stage of females plays a major role in mating behaviour in which the fully ripe ovary females are highly attractive to the dominant BC males compared to those females at very early stages of ovarian ripeness. Ovarian maturation of prawns is strictly associated with moulting such that females with fully ripened ovaries have a higher chance of moulting than females with un-ripened ovaries. Thus, it is suggested that BC males respond according to ovarian maturation status of females rather than to their moult stage. However, as a rule of thumb, it can be suggested that the moult stage of female prawns is a valuable indirect indicator of ovarian maturation status. Surprisingly, the interpretation of sexual attractiveness behaviour of crustaceans in most previous studies that focused mainly on moulting stage parameters in mating attraction where as this study indicates that more attention should be paid to the ovarian maturation status of females instead.

3.6.2 The effect of male's moult stage on the sexual attraction behaviour response of newly-moulted females.

Newly-moulted female *M. rosenbergii* responded more rapidly to both pre- and inter-moult males than newly-moulted males. Similar findings by Parnes *et al.* (2006) who reported that sexually mature pre-moult male shrimp *L. vannamei* were not attractive to the receptive females. They related such behaviour to the fact that spermatophores of those pre-moult males disappear from the terminal ejaculatory ducts of males 12 - 24 h prior to moulting.

From the previous literature and from the current study it can be suggested that pheromone release is strictly associated with the moult cycle. Varying sexual responses by newly-moulted female prawns to different moult stages of mature BC males indicates that different pheromone contents were released from the BC male throughout their moult cycle. Findings here suggest that receptive female *M. rosenbergii* can detect pheromones released from the BC males and they can distinguish the appropriate male for mating. Also, in order to mate with a proper and strong BC male, newly-moulted female prawns have to avoid soft shell male and to ensure paternity, they have to seek for a proper BC male who can provide protection.

While there seems to be an association between attraction behaviour by BC male and ovarian maturation status of female, a similar relationship could exist with BC males where the presence of spermatophores could be influencing sexual attraction more than the moult cycle of BC males. This may explain why the receptive newly-moulted female prawns were more attracted to both the inter- and pre-moult stages than the newly-moulted BC males. Another possible reason could be that the amount of pheromone released from newly-moulted BC males is either inadequate or absent. Also, there is a possibility that the newly-moulted female prawns avoid coupling with soft shell BC males because they are not strong enough to protect them from predation. Thus, it can be suggested that moult cycle of the BC males *M. rosenbergii* has a significant influence on attracting the most receptive newly-moulted females.

As in the majority of previous studies concerning mate choice and sexual responses in Crustacea which claimed to be caused by the receptive of female moulting, results

obtained here suggests that ovarian maturity of female prawns is playing a high potent role in sexual attraction behaviour of BC male. Thus, the maturity status of female's ovaries is a more substantial factor influencing sexual responses than moulting.

3.6.3 The effect of male morphotypes on sexual attraction behaviour of females.

All newly-moulted female prawns responded to the three male morphotypes but with varying levels of attraction. Newly-moulted females were seen to respond significantly more rapidly to the dominant BC male than to subordinate SM and OC males and similarly responded faster to SM than to OC males. Then newly-moulted female preference for dominant BC males is not surprising since these BC males have higher fertilisation potential than subordinate SM and OC males and can ensure undisturbed spawning (Ra'anana and Cohen, 1983). Also, BC males provide more protection than both SM and OC males. Similar preferences of females with respect to males with sexual size-dimorphism have been reported for the lobster, *H. americanus* (Bushman and Atema, 2000) who noticed a high preference of females to the dominant males for mating over the sub-ordinate males. Also, the rock shrimp *R. typus* females had preferences for robustus (dominant males) over typus (sub-ordinate males) during mating (Correa *et al.*, 2003; Díaz and Thiel, 2003; 2004; Hinojosa and Thiel, 2003).

However, evidence in this study that BC male prawns for *M. rosenbergii* emit sex pheromone, which attracts newly-moulted female is in contrast to the findings of Hartnoll and Smith (1979) who concluded there was absence of sex pheromone released from male edible crab, *C. pagurus*. Their conclusion was based on male negative sexual behavioural response when exposed to another male but not to a female where in the

current study, the conclusion was based on sexual behavioural of receptive female responding to the different male morphotypes. Also, Hartnoll and Smith's (1979) conclusion was based on an assay of male cardiogram experiment in which they implanted electrodes devices on either side of the heart of mature inter-moult males. They found no consistent changes in heart rate beating after exposing them to urine collected from paired pre-moulted females. Technically, in the current study the behavioural responses were tested directly by using a bioassay system while the study conducted by Hartnoll and Smith (1979) tested the behavioural responses of male edible crab, *C. pagurus* indirectly by measuring the heart rate beating. Naturally, adult *M. rosenbergii* have several moult and gonadal maturation cycles within one year while the *C. pagurus* has only one annual moult cycle which is during breeding season. This may cause such difference in sexual behavioural responses between the two studies.

The results of this study is supporting previous findings by Díaz and Thiel (2003; 2004) who found females *R. typus* had preference for dominant males during mating more than the sub-ordinate males. However, the experimental conditions of their studies were slightly different from the current study. First, they restricted the movement of the males by tying a fine nylon thread around their cephalothorax and abdomen which may have caused stress or physical restriction and accordingly affected their behaviour towards females. Also, they used a Y-maze design bioassay in which along with chemical communication cues, both visual and tactile cues may also have been involved. Thus, attraction behaviour seen between male and females was not necessarily due to chemical cues. Furthermore, since they used standing water (not running water) in their experiment, this may have limited chemical cue transfer between individuals.

Also, similar observations have been reported previously by Bushmann and Atema (2000) who noticed that female lobster *H. americanus* have high preference to mate with dominant males over the sub-ordinate males.

The work presented in this chapter illustrates clear differences in behaviour according to moult stage and morphotype. The female's ovarian maturation is a key component in the mating behaviour of *M. rosenbergii*. Newly-moulted female prawns are the most receptive stage due to their completed ovarian maturation hence releases pheromone(s) to attract males. Since there are slight sexual responses displayed by both pre and inter-moult females toward the BC males, it can be suggested that newly-moulted female *M. rosenbergii* release certain pheromone(s) into the water, more likely via the urine, which attract BC males.

Several previous studies reported that adult receptive female crustaceans may choose their mate based on male characteristics indicative of his fitness, secondary sexual characters, courtship behaviour, size and quality of territory, amount of mating, and / or genes (Kamiguchi, 1972, Thornhill and Alock, 1983, Jackson and Elwood, 1989). In addition to the above characters, results in this study suggest that female can select male based on males' moulting stage. Male, in contrast, has been reported to choose female for mating based on body size and fecundity as has been reported in some crustacean species such as the freshwater prawn, *P. paucidens* (Kamiguchi, 1972), the spider crab, *Inachus phalangium* (Diesel, 1988), the shore crabs *C. maenas* (Reid *et al.*, 1994) and the amphipod, *Gammarus pulex* (Elwood *et al.*, 1987). Also, the time remaining until spawning is another factor that can cause a male crustacean to choose a female as has

been noticed in the spider crab, *I. phalangium* (Diesel, 1988), the fiddler crabs, *U. lacteal* (Goshima and Murai, 1988) and *U. tetragonon* (Goshima *et al.*, 1996). Beside the above factors, results of the current study can suggest that ovarian maturation and moult stages are additional important key factors for BC male prawns selection for mating. In the enclosures, because newly-moulted female prawns prefer BC males over OC and SM morphotypes, suggesting that the most dominant males have more advantageous in guarding and mating. Thus, male morphotype of *M. rosenbergii* is an important factor in female's sexual attraction behaviour. However, the present finding in this study is contrary to the findings reported by Jivoff and Hines (1998) who found no difference between the male sizes of blue crab, *C. sapidus* in guarding and copulation and no significant difference in females preference.

Pheromone release by sexually mature males is also important in the sexual attraction behaviour of *M. rosenbergii*. Pheromones released from the BC males are probably differing from those released by SM and OC male morphotypes. This explains why newly-moulted females have higher preference for the most dominant males (BC males) over the other male morphotypes. Thus, it can be suggested that sexual attraction behaviour in the freshwater prawn *M. rosenbergii* clearly reflects the expected dominance hierarchy and must be under substantial chemical control as the males in this study were prevented from the use of visual cues. Also, it can be concluded that male mate selection in *M. rosenbergii* is based largely on the detection of female pheromone compounds. It is interesting to speculate whether this reflects differences in pheromone output (concentration) or pheromone identity between morphotypes. Because BC males

were the most attractive morphotype for all females, hence their urine will be the key target investigated in following chapters.

Chapter 4 : The effect of sample treatment on sex pheromone efficiency in sexual attraction

4.1 General introduction

Chemical communication has been shown to influence behaviour of various crustaceans (Ryan, 1966; Caldwell, 1979; Seifert, 1982). Chemical senses appear to be the most important to play major roles in regulating some behaviour of many decapods crustaceans in various social interactions, location and evaluation of food and navigation and evaluation of habitat (Rittschof, 1992). Also, chemical signals are used for both mate location and attraction (Gleeson, 1991) as well as for evaluating the quality or reproductive state of the sender (Jones and Hartnoll, 1997). Numerous studies on decapod crustaceans have demonstrated the importance of chemical communication signals during reproductive behavioural interactions (Ryan, 1966; Atema and Enstrom, 1971; Eales, 1974; Gleeson, 1980).

While communication between individual crustaceans has been studied extensively, investigations into the role of chemical signals between male–female for reproductive interactions are lacking. As demonstrated, in the previous chapter, about the effect of moulting stage and male morphotypes on sexual attraction behaviour which then proved the existence of chemical cues released from *M. rosenbergii* in water. Thus, more than one chemical substance might be involved in controlling chemical communication between adult male and female *M. rosenbergii*. Literature suggested different source(s) of these chemicals in several aquatic crustaceans where urine is one of those and most candidate sources. Furthermore, more than one chemical substance might be involved in controlling chemical communication between adult males and females *M. rosenbergii*.

It has been shown that urine in crustaceans is a liquid that can carry important chemical information in intra-specific interactions between individuals such as for courtship and mating behaviour (Ryan, 1966; Christofferson, 1978; Bushmann and Atema, 1994; 1997; 2000; Atema, 1995; Karavanich and Atema, 1998b; Kamio *et al.*, 2000; Hardege *et al.*, 2002; Raethke *et al.*, 2004). Also, urine in an aquatic crustacean plays in recognition of social hierarchy status of the opponent and identifying other species of crustacean (Karavanich and Atema, 1991; 1998a, b; Breithaupt *et al.*, 1999; Zulantz-Schneider *et al.*, 2001; Breithaupt and Eger, 2002). Horner *et al.* (2006) investigated the source and specificity of the aggregation signal in Caribbean spiny lobsters, *Panulirus argus*. They found that urine from either sex is involved in mating between both males and females spiny lobsters. They suggested that those cues are mediating copulation behaviour and showing that urine is at least one source of the aggregation signal. Raethke *et al.* (2004) has shown that pheromones are released with the urine and that spiny lobster *J. edwardsii* post-moulted females during reproductive period were capable of distinguishing between male and female urine and were strongly attracted to male urine. They suggested that pheromones emitted with the males' urine helps females to locate and chose a suitable mate.

Controlling urine release may affect communication profile in aquatic crustaceans. The behavioural response to chemical signals in urine may also change depending on the urine volume presented. In other words, aquatic crustacean individuals may respond to each other according to the amount of urine received from the surrounded environment. Several attempts of studying behavioural responses of aquatic crustacean after restricting urine release whether by controlling the amount of urine or by blocking the

urine opening. Bamber and Naylor (1997) observed that females were less attractive to males following blocking of antennal gland opercula of shore crab, *C. maenas* males. When male lobsters, *H. americanus* urine nephropores were blocked, Bushmann and Atema (2000) noticed a significant reduction in the incidence of female shelter approach and the time spent attempting to enter. In a comparison of agonistic behaviour study in blocking and unblocking opercula of crayfish, *O. rusticus*, Schneider *et al.* (2001) found that the presence or absence of a chemical borne in urine plays a significant role in recognition between crayfish individuals. They found crayfish individuals fight longer time and more intensely when urine cues were absent (blocked opercula) than when urine cues were present (unblocked opercula). They concluded that communication of behavioural states through urine plays an important role in social recognition.

It is clear that urine in many aquatic crustaceans plays a significant role in chemical communication, especially for reproductive behaviours. At present, we know virtually nothing about the chemical identity or the release dynamics of the aggregation cue contained within the urine of *M. rosenbergii*. Although previous works indicated that the quantity of urine released by individual crustacean is playing a considerable factor in the sexual interaction, however, there are very limited studies on testing urine volume (concentration) for sexual behavioural attraction. Generally, some of previous studies were focused on the duration and the frequency of urine released from crustaceans into the water environment. Simon and Moore (2007) studied the length period of time that both male and female crayfish *O. rusticus* emitting urine. They noticed, significantly, the highest amount of urine (91%) and the number and the duration of urine released by

both male and female crayfish *O. rusticus* were when they were in a reproductive stage. Others, in an agonistic behavioural study, Bergman *et al.* (2004) found that during agonistic encounters, the dominant crayfish *O. rusticus* release urine more frequently than sub-ordinates.

In contrast, some previous studies showed that crustacean species has limitation of detecting others chemicals. A study conducted by Bushmann and Atema (2000) on lobster *H. americanus* over a period of 12h, where both dominant (strongly preferred by the female) and sub-ordinate (less preferred) males placed together along with the female lobster *H. americanus*, the quantity of urine collected from both males had no significant difference. They suggested that any discriminatory cue is likely to be qualitative rather than quantitative in nature.

4.2 Urine concentrations

From previous studies, it is clear that aquatic crustaceans use chemicals for necessary communication, with urine being a common carrier of such information with respect to e.g. information about an individual's gender, reproductive status, and possibly reproductive readiness or receptivity. Individual communicators are likely to control the amount of urine released into the surrounding environment as a part of their behaviour, this depending on the receiver's social and/or maturation status. The amount of urine released may have an influence on the attraction behaviour of aquatic crustacean. In other words, the length of time for attraction behaviour response of individuals takes may depend on the volume or concentration of urine coming from the sender. However,

the question of what level of urine volume can cause positive sexual attraction responses is still vague and needs further investigation.

The literature is very poor and limited in terms of studies investigating the effect of urine concentration of aquatic crustaceans on chemical communication. Surprisingly, studies on the effect of using different urine concentrations on the reproductive communication behaviour of aquatic crustaceans are scarce. In particular, research to establish the minimum urine concentration that could cause a positive attraction response is very poor for aquatic crustaceans. Amongst those that do exist, a study conducted by Atema and Cowan (1986) who observed a positive sexual behaviour response in American lobsters, *H. americanus*, when a urine volume of 500 μ l and waterborne body odour volume of 20 ml were introduced to a test chamber. Others, Bushmann and Atema (2000) studied the effect of urine volume collected from female lobster *H. americanus* on male sexual behaviour and found that males increased urine output in response to females attempting to enter their shelters. Horner *et al.* (2006) studied chemical cues by using collected urine from both male and females of spiny lobsters *P. argus* that mediated shelter preference. Another study conducted by Ekerholm and Hallberg (2005) who delivered urine from pre-moult female *C. maenas* at different concentrations in far, near, and close/contact ranges, either as a pump-generated plume or deposited on a polyurethane sponge. They found that substances in pre-moult female urine function as primer and potent short-range releaser pheromones. They concluded that the stimulus from pre-moult females is sufficient to elicit increased search and mating-specific behaviours. They suggested that there is a certain level of pheromone which makes *C. maenas* responds to each other. In other words, they stated that there is at least a

minimum level of pheromone which is enough to let shore crabs show a behavioural response. However, they reported that pheromone concentrations play a role in male acceptance of females, recruiting more males to respond and generating better responses with increasing concentration. Such studies showed the importance of urine as a carrier of chemical cues for chemical communication among individuals in aquatic crustacean populations.

For freshwater prawns, especially *M. rosenbergii*, studies of urine concentration affecting reproductive behavioural responses remain to be carried out. Such research is therefore essential in order to assist the understanding of chemical communication between *M. rosenbergii* individuals. Several important aspects need to be investigated concerning urine characteristics and its influence on attraction behaviour of *M. rosenbergii*. First, is the question of whether urine concentration obtained from *M. rosenbergii* is playing any role in the sexual behavioural responses between individuals? If so, at what approximate level does it act? Second, how stable are the attractive components of urine and therefore do different treatments (cold storage, freezing and heating) affect urine quality and efficiency in terms of attraction behaviour? Finally, how long will it take to attract *M. rosenbergii* and if a combination of these factors (concentration and temperature) were applied to the fresh urine. To answer the above questions, behavioural experiments were designed and data were then collected and analysed.

4.3 Urine temperature treatment

One aspect of this study is to investigate the characteristics of sex pheromones of freshwater prawn *M. rosenbergii*. Urine along with other excreted chemicals plays an important role in courting and mating behaviour in many animal taxa, including aquatic crustaceans. Along with water, those excreted chemical contains several metabolite end products of some organic substances such as proteins and carbohydrates and some other inorganic substance such as minerals. Sex pheromones are probably made up from a complex of several substances carried within urine. In many animal species, including aquatic crustaceans, breeding usually take place when surrounding temperature reach an appropriate level. This means that sexual detection behaviour varies across the year according to the temperature. The stability of the pheromones with respect to temperature may therefore have a direct affect on sex pheromone quality and efficiency during sexual attraction.

Accordingly, a number of trials were carried out to investigate the effect of different treatments on urine attractiveness, which in turn reflects the stability / friability of pheromone components contained in the urine. It was hypothesized that exposing fresh urine to different temperature treatments could change the characteristics of those organic substances. In other words, would temperature treatment of sex pheromone affect their efficiency in sexual attraction behaviour? If so, to what extent? To test this hypothesis, urine was exposed to different temperature treatments such as cooling, freezing and heating. In order to explore and understand more of urine efficiency in reproductive attraction, fresh urine of BC male *M. rosenbergii* was exposed to varied temperature treatments (cooled, frozen and heated).

Very few studies have been carried out on the interaction of temperature treated sex pheromones on sexual attraction behaviour. A number of studies examine responsiveness to pheromone at different temperatures, although this may be presumed to be less dependent on pheromone friability than responsiveness of the receiver. For instance, Linn *et al.* (1988) showed that the response specificity of oriental fruit moths *Pectinophora gassypiella* and pink bollworm moths *Grapholita molesta* males to sex pheromone specificity was temperature-dependent. For both species, Linn *et al.* (1988) observed that at 20°C, males exhibit a narrow pheromone blend-dose of specificity while at 26°C, however, males exhibit a significantly lower degree of specificity. They concluded that the degree of response specificity can be significantly affected by temperature.

In freshwater teleosts, Bhatt *et al.* (2002) studied the influence of both water temperature and pH on olfactory sensitivity in male Hamilton's barila *Barilius bendelisis*. They tested sexual responses of male *B. bendelisis* to two different pheromones extracted from females (pre-ovulatory steroid sulphate and post-ovulatory 15-keto-prostaglandin) at two different water temperatures at 16.5°C (during pre-spawning season) and at 23°C (during spawning season). At 16.5°C and in observation aquaria, the 20 ml min⁻¹ of water flow rate and the 300 ml solution of from both pre-ovulatory and post-ovulatory pheromones concentrations, they did not notice any significant difference in the male response to both of the female pheromones compared to the control (blank water). However, Bhatt *et al.* (2002) observed that male sexual responses at 23°C were significantly higher than the controls under similar experimental conditions.

4.3.1 Cooled urine treatment

The literature contains a very limited number of studies on testing the effect of storing urine (or sex pheromones) at low temperatures on sexual attraction behaviour, especially for aquatic crustaceans, although the effect of temperature on pheromone production has been studied. Under laboratory conditions, Raina (2003) conducted an experiment to study the effect of different temperatures on sex pheromones, but not urine, production in corn earworm *Helicoverpa zea*. He tested the effect of three temperature treatments (15, 25 and 35°C) on the sex pheromone concentration in the female corn earworm. Among his observations, Raina (2003) noticed that under normal temperature (25°C) conditions, females produced an average of 36.1 ± 3.9 ng, while cool (15°C) temperature treatments dramatically reduced the sex pheromone concentration produced in females (2.8 ± 1.1 ng/female). Depending on the nature of pheromone components, it is possible that behaviour activity will decline with storage with respect to the activity observed in fresh urine. This may occur through degradation of the compounds or through loss from urine by adsorption onto holding containers or loss of volatiles in the air. This will clearly not occur if the compounds are highly stable, non-volatile or non-adsorptive (“sticky”).

4.3.2 Frozen urine treatment

Very little research on using frozen urine in sexual response behaviour were found. This shortage of such studies and others investigating pheromone stability, is one of the challenges and difficulties in getting a comprehensive understanding regarding the key attractive substances and their roles in chemical communication, especially for sexual attraction behaviour.

Horner *et al.* (2006) examined the source and specificity of the chemical signals mediating sheltering behaviour in Caribbean spiny lobsters *P. argus*, by measuring the time spent inside their shelters and the numbers of entries into the shelters. Horner *et al.* (2006) study conducted a study of the preferences of spiny lobsters to shelters emanating conspecific urine signals by using a bioassay system flume (12 L, 0.75 W and 0.35 m H) containing 2500 l of seawater and water flow rate of 300 ml min⁻¹ (5 cm/s). They collected urine from sub-adult (young adults) male and female spiny lobster by connecting catheterised tubing to their nephropore ends with plastic vials. Collection vials of urine were emptied daily, pooled and stored at -20°C. Urine stimuli in the sex-specific experiment consist of either pooled urine collected from all 4 catheterised males or from all 4 catheterised females. Prior to each trial, urine was thawed and diluted 1:10 or 1:100 in artificial seawater taken directly from the flume. Observation period for each trial was 1h and the lobster behaviour was recorded by a video camera mounted above the bioassay tank. Urine solution stimuli were pumped into the bioassay flume at about 15ml h⁻¹. This means that the 1:10 and 1:100 dilutions are about 1500 and 150 µl respectively, of urine concentration released into the flow over the course of 1h. Compared with the control (only seawater), results of their study had showed that both male and female spiny lobsters responded to both the 1500 and 150 µl urine concentrations of conspecific urine pooled across sexes. Furthermore, they suggested that very low volumes (less than 150 µl) of conspecific urine are sufficient to elicit attraction behaviour of spiny lobster *P. argus* and they strongly suggested that urine is at least one source of communicational signal in this species.

In another study, Bamber and Naylor (1997) studied the level of male shore crabs, *C. maenas* threshold detection of pheromone to pre-moult female urine. They used an octograph system to assess chemical communication between crabs on a real time basis. They collected and pooled urine from fifteen pre-moult females and then saved it in a freezer at -20°C. At the time of each trial, the urine was thawed and a number of serial dilutions were made in filtered seawater to give urine volumes of 0.1, 1.0, 10 and 100 µl to be tested against 29 males. They noticed that the sexual responses of male shore crabs *C. maenas* increased with increasing urine concentrations (0.1, 1, 10 and 100 µl l⁻¹ of filtered seawater) that had been collected from pre-moult females. Bamber and Naylor (1997) demonstrated that around 90% (26 out of 29) of the males have showed positive sexual responses to a dilution of 100 µl l⁻¹ filtered seawater while less than 14% (4 out of 29) of males showed a positive sexual response to 0.1 µl l⁻¹ filtered seawater.

Many pheromones and semiochemicals of different animal species exist in the environment in different concentrations, such that degradation in the environment over time may not be a serious barrier to effectiveness. For instance, Bjerselius *et al.* (1995) studied the behavioural response of male goldfish *Carassius auratus* to the sex pheromone 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) in the ambient water at concentrations of 10⁻¹¹ mol l⁻¹. The authors noticed that fish spent significantly less time in water scented with 17α,20β-P than in control water and spontaneous locomotor activity increased significantly. Furthermore, studies on sex pheromones of some insects such as moth species show activity at concentrations of 10⁻¹⁸ Mol (Wyatt, 2003). For mammalian species such as mice, vomeronasal neurons are sensitive to some pheromones at concentrations of 10⁻¹¹M (Leinders-Zufall *et al.*, 2000). Furthermore,

pheromones concentrations released from larval and adult lamprey are present in the environment media as low as 10^{-12} M (Li *et al.*, 1995; Polkinghorne *et al.*, 2001).

In addition to its potentially preserving properties, freezing may also be responsible for degradation of activity, especially when material is subjected to cycles of freeze-thawing. Freezing will therefore be employed to assess the friability of pheromone compounds in the urine.

4.3.3 Heated urine treatment

The literature is extremely poor with respect to any studies testing the effects of heated urine of aquatic crustaceans with respect to attraction behaviour. However, a single study has tested the effect of boiled conditioned water, but not urine, on the ovary maturation of the freshwater prawn *M. kistnensis*. Nagabhushanam *et al.* (1989) tested the influence of the presence of male freshwater prawns, *M. kistnensis*, on the female's ovary maturation. Comparing between two conditioned water sources, water collected from a tank where sexually mature females were reared in isolation and water collected from a tank where both sexually mature females and male *M. kistnensis* had been kept, they found that the latter type of water had an effect on the female's ovary maturation. This means that conditioned water held chemical cues secreted from the sexually mature male prawn which were important to ovary maturation of females. However, when such conditioned water was boiled, Nagabhushanam *et al.* (1989) found no evidence of any influence on female ovary maturation, suggesting that boiling had destroyed the active component.

Finding of the previous chapter showed the existence of sexual pheromone in the water which is released from the adult and mature prawns in which play an important role in sexual attraction. Along with water, urine is likely the main carrier of sexual pheromones which contains several and complex biological chemical substances such as peptides, carbohydrates and minerals. As found in the previous chapter of being the BC males *M. rosenbergii* are the most attractive to the newly-moulted females, however, the effect of fresh urine volume collected from the BC males on sexual attraction is still unknown. Subsequently, a question raise is the volume of fresh urine is a limiting factor in sexual attractions between adult males and females *M. rosenbergii*. If so, what is the limiting urine volume can make a significant sexual response?

On other hand, content profile of pheromones in the freshwater prawn *M. rosenbergii* is anonymous. Since many proteins / peptides are recognised to denature above 60°C, urine was subjected to heating at 70°C to examine the effect of heating on degradation of activity with the intention of better characterising the active pheromone component. So, studying some of their characters may give guidance to more understanding of their nature. For instance, exposing urine to different temperature may affect the nature and then the efficiency of the pheromones, if so, to what extent? Treating urine with wide ranges of temperature of cooling, freezing and heating is possible to answer this question. Thus, there are two main objectives in this chapter: first, is to study the effect of fresh urine volume collected from BC males on the sexual attraction behaviour. Second, the effect of temperature on the quality of urine collected from prawn in the sexual attraction.

4.4 Materials and methods

The previous chapter showed that newly-moulted female prawns with fully ripe ovaries are the most receptive and attracted to water-borne cues derived from the most dominant male BC, from amongst the male morphotypes. In this experiment, urine was therefore collected from BC males in order to test the sexual attraction behaviour responses of newly-moulted females. The ability of *M. rosenbergii* to discriminate between conspecific urine signals at different volumes will be examined in the following study. When newly-moulted female(s) became available therefore, fresh urine was collected from several BC males on a daily basis.

4.4.1 Urine collection

When urine was needed for an experiment, large mature BC males were collected from the holding tanks. Each BC male was anaesthetised (see section 2.2.9 on page 67 and section 2.2.10 on page 68) and then urine was collected (as described in section 2.2.9 on page 67). The collected fresh urine, from several BC males, was pooled and kept inside clear Eppendorfs (1.5ml each). From these Eppendorfs, different fresh urine volumes, depending to the experimental design, were collected using an adjustable volume micropipette (single channel) and injected into the source chamber (A) of the bioassay tank system according to the designated trial design.

4.4.2 Experimental bioassay tank set up

Prior to each experimental trial, the water flow, water temperature and oxygen levels of the experimental tank were fixed and recorded (as described in section 2.2.4 on page 60). For each trial, a newly-moulted female was placed for acclimation in the test

chamber (B) of the bioassay system for 30 minutes and each experimental trial lasted 30 min afterwards.

4.4.3 Behavioural response with fresh urine

Early morning, whenever any newly-moulted female was found, fresh urine from BC males was collected. Six volumes (10, 100, 200, 300, 500 and 1000 μl) of fresh urine were injected into the source chamber (A) of the bioassay tank. Since each of bioassay tanks contains approximately 100 litres so, adding 10, 100, 200, 300, 500 and 1000 μl volumes of urine which are fully dispersed would give a nominal concentration of 0.1, 1.0, 2.0, 3.0, 5.0 and 10 $\mu\text{l l}^{-1}$, respectively. To test the sexual attraction behaviour of newly-moulted females *M. rosenbergii* placed in the test chamber (B) of the experimental bioassay tank. This procedure was run five times a day with, 1 control run, 2 forward and 2 backward tank orientations conducted for each female. Twelve newly-moulted females were used for each fresh urine volume.

4.4.4 Temperature treatment

For all temperature treatments (fresh, cooled, frozen and heated), fresh urine was collected from BC males and treated with different temperature regimes (see section 2.2.11 page 68). For the cooled treatment, fresh urine was stored in a fridge at 4°C for 24-72 hours before being used for the sexual attraction behaviour test. Similarly, some of fresh urine was stored in a deep freezer at -70°C for at least 72 hours before being used in the experiment as frozen urine. The heated urine was prepared by holding fresh urine inside Eppendorfs and placing it in a water bath at 70°C for 1 hour. For each of

these temperature regimes, different concentrations (0.1, 1.0, 2.0, 3.0, 5.0 and 10 μ l l⁻¹) were tested for the sexual attraction behaviour of newly-moulted females.

As mentioned above (see section 2.2.6 on page 62) after each trial, the experimental tanks were cleaned and washed, especially the internal side walls, to avoid any odour residues from the previous trial. All sexual response behaviours were video taped, analysed and reported.

4.5 Statistical testing

A hypothesis can be proposed that applying any fresh (recently collected) urine volume will not affect the sexual attractiveness range. So, similar sexual response is expecting from newly-moulted female when any fresh urine volume is injected in the bioassay system. Also, it can be proposed that the efficiency of fresh urine collected from the BC males *M. rosenbergii* in sexual attraction will not change if exposed to vary range of temperature. In order to test these hypotheses, two different trials were designed, one to test the effect of different volumes of fresh BC urine on the sexual attractiveness responses and the other is to test the effect of the fresh BC urine exposed to various temperature treatments.

Results were analysed using a repeated measures general linear model (GLM). For analysing the effects of fresh urine collected from BC males on the time taken to reach the target by newly-moulted females (this trial employing 6 urine volumes in addition to the control), a 2 (trials) \times 2 (test chamber orientations) \times 7 (urine volumes including 0

control) design was employed. For analysing all samples together (the remaining samples having no $0.1 \mu\text{l l}^{-1}$ concentration (volume of $10 \mu\text{l}$) urine concentration and therefore only 6 urine concentrations), a 2 (trials) \times 2 (test chamber orientations) \times 6 (urine volumes including 0 control) design was employed.

Post-hoc tests were carried out using Tukey's HSD test for differences between individual samples and a Dunnett's test for differences between all samples and a control group (all tests checking to see if the test group is less than the control group).

4.6 Results

Before presenting the results part of this study, it is worth mentioning the effect of the bioassay tank direction (orientations) during the female's behavioural response trials. Since each newly-moulted female prawn was used five times in each trial, (1 for control and 4 for test, 2 forward and 2 reverse orientations), a statistic test (see section 4.5 page 130) was ran to test the experimental bioassay tank orientations. Statistical analysis results showed no significant difference ($p < 0.05$) neither in the experimental run nor in tank orientation directions in any of urine concentration used see Table 17 and Figure 17.

4.6.1 Effect of urine concentration on the time taken by newly-moulted females to reach target.

Urine concentration is considered here separately from the temperature treatments as there is an additional $0.1 \mu\text{l l}^{-1}$ sample but this data are also included in the later analyses with the non-significant $0.1 \mu\text{l l}^{-1}$ sample omitted. Table 17 shows repeated measures GLM for responses to urine concentration and indicates that concentration had a significant effect on response time. Experimental run number and orientation of the test chamber were not significant from this analysis (Figures 18 and 19). In this experiment it was clear that higher volumes of fresh urine gave faster newly-moulted female response times.

Table 17: Repeated measures GLM for effects of fresh male blue claw urine on the time taken by newly-moulted females to reach target. Run = Experimental run, Orient = Chamber orientation, Vol. = Urine concentration. Bold indicates $p < 0.05$.

	SS ¹	DF ²	MS ³	F	P
Intercept	182122.2	1	182122.2	6286.977	<0.000001
Error	318.6	11	29.0		
RUN	1.6	1	1.6	0.099	0.758653
Error	174.5	11	15.9		
ORIENT	5.1	1	5.1	0.360	0.560868
Error	154.5	11	14.0		
VOL	16870.0	6	2811.7	193.878	<0.000001
Error	957.1	66	14.5		
RUN*ORIENT	1.6	1	1.6	0.051	0.825185
Error	344.3	11	31.3		
RUN*VOL	320.7	6	53.4	2.214	0.052429
Error	1593.0	66	24.1		
ORIENT*VOL	225.5	6	37.6	1.287	0.275491
Error	1927.4	66	29.2		
RUN*ORIENT*VOL	207.0	6	34.5	1.157	0.339862
Error	1967.7	66	29.8		

¹ Sum of Squares

² DF: Degree of Freedom

³ Mean of Squares

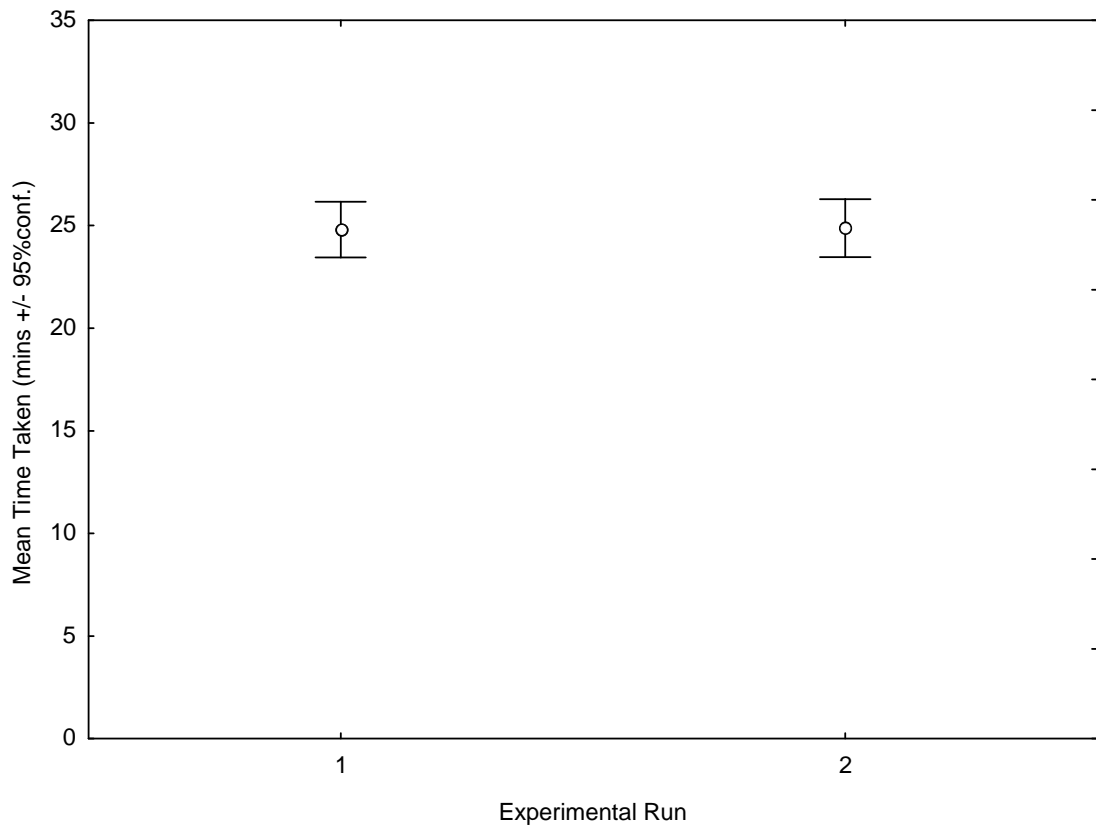


Figure 17: Graph of the mean time taken by newly-moulted females to reach target exposed to fresh male blue claw urine between the first and the second trial runs.

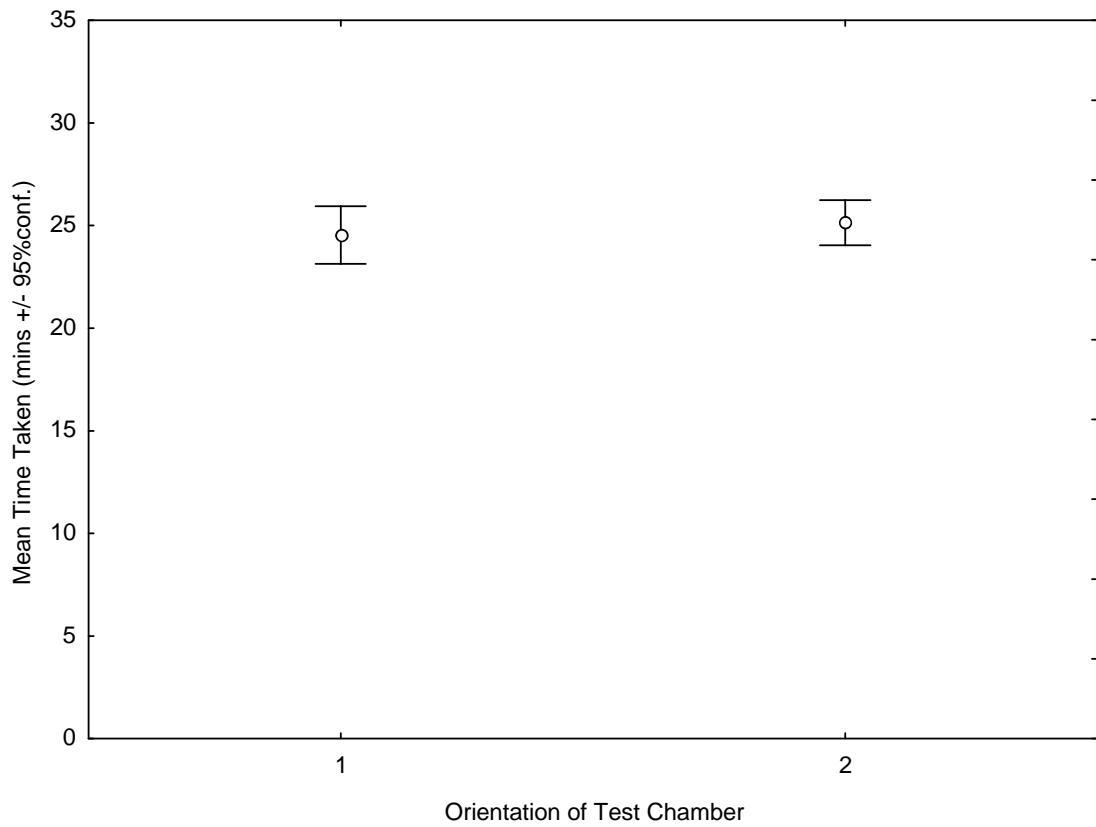


Figure 18: Graph of the mean time taken by newly-moulted females to reach target exposed to fresh male blue claw urine for forward and reverse tank directions.

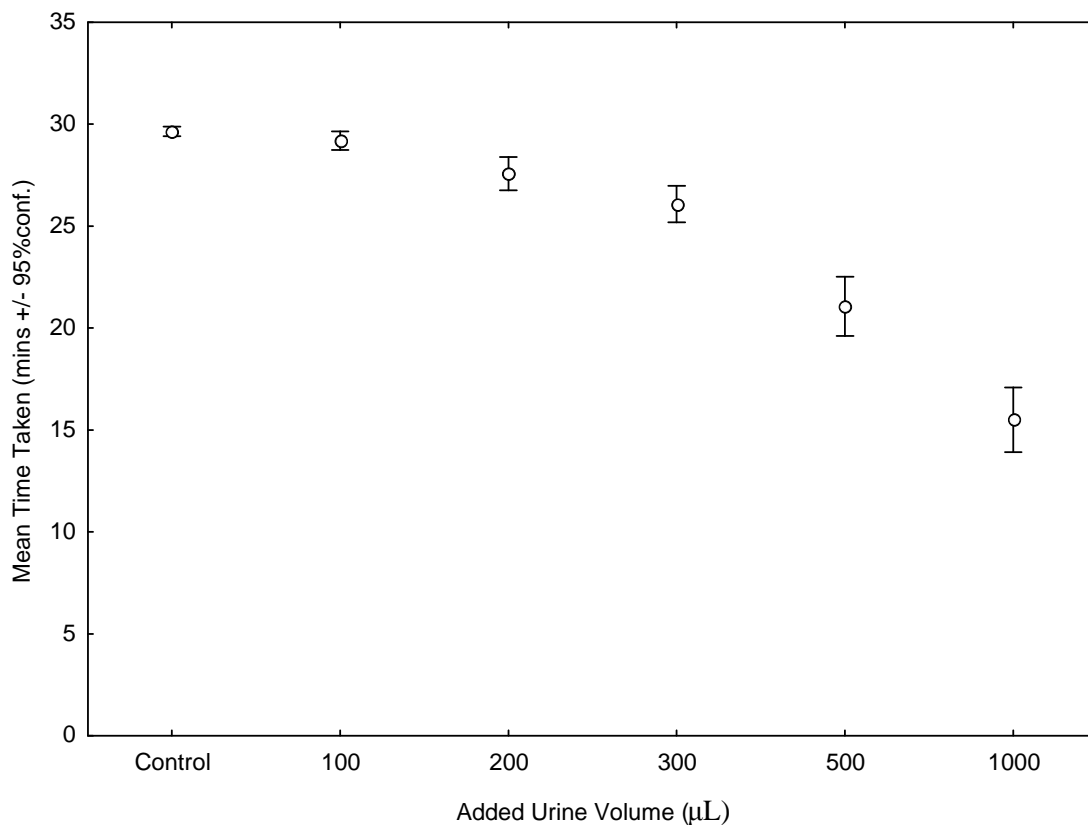


Figure 19: Graph of the mean time taken by newly-moulted females to reach target exposed to different concentrations of fresh male blue claw urine, $n = 12$.

Table 18 shows a Tukey HSD *post-hoc* analysis of the data of fresh BC urine. From this it can be seen that responses to the 0.1, 1.0 and 2.0 $\mu\text{l l}^{-1}$ samples are not significantly different from those seen by the controls (the more accurate Dunnett's test, Table 19 however, suggests that the 2.0 $\mu\text{l l}^{-1}$ is also significantly different). All other concentrations more than 2.0 $\mu\text{l l}^{-1}$ are significantly different from one another.

Table 18: Tukey HSD *post-hoc* test for overall the time taken by newly-moulted females to reach target exposed to fresh blue claw male urine. Bold indicates $p < 0.05$, $n = 12$.

Urine volume	Control	0.1 $\mu\text{l l}^{-1}$	1.0 $\mu\text{l l}^{-1}$	2.0 $\mu\text{l l}^{-1}$	3.0 $\mu\text{l l}^{-1}$	5.0 $\mu\text{l l}^{-1}$	10.0 $\mu\text{l l}^{-1}$
0.1 $\mu\text{l l}^{-1}$	0.999989						
1.0 $\mu\text{l l}^{-1}$	0.998272	0.999931					
2.0 $\mu\text{l l}^{-1}$	0.103467	0.169847	0.304592				
3.0 $\mu\text{l l}^{-1}$	0.006941	0.013736	0.032764	0.954161			
5.0 $\mu\text{l l}^{-1}$	0.000128	0.000128	0.000128	0.000130	0.000258		
10.0 $\mu\text{l l}^{-1}$	0.000128	0.000128	0.000128	0.000128	0.000128	0.001095	

Table 19: *Post-hoc* Dunnett's test against control for overall the time taken by newly-moulted females to reach target exposed to fresh male blue claw urine. Significance denotes that time taken to reach target is less than control. Bold indicates $p < 0.05$, $n = 12$.

Urine volume	Probability that time taken to reach target is less than control
0.1 $\mu\text{l l}^{-1}$	0.786599
1.0 $\mu\text{l l}^{-1}$	0.664571
2. $\mu\text{l l}^{-1}$	0.018989
3.0 $\mu\text{l l}^{-1}$	0.001088
5.0 $\mu\text{l l}^{-1}$	0.000021
10.0 $\mu\text{l l}^{-1}$	0.000021

4.6.2 Effect of urine concentration and urine temperature treatment on the time taken by newly-moulted females to reach target.

The interaction of urine concentration and temperature treatment type was studied and the results are presented below. Since there no significant found for the $0.1 \mu\text{l l}^{-1}$ of fresh BC urine to attract newly-moulted female prawns, thus, it not encouraging to repeat this particular volume with temperature treated urine. So, the analysis here includes the foregoing data set but with the $0.1 \mu\text{l l}^{-1}$ concentration samples omitted to give a balanced design.

Table 20 shows repeated measures GLM of the responses of the newly-moulted female prawns to different concentrations and temperature treatments of male BC urine. From this analysis it can be seen that temperature treatment and concentration added both had a significant effect on the response of females as did the interaction between these two main effects. Surprisingly, tank orientation also had a significant effect with interactions being seen with treatment, run and the concentration of urine being added. Run number did not have a significant effect of itself.

Table 20: Repeated measures GLM for effects of fresh male blue claw urine on the time taken by newly-moulted females to reach target. Run = Experimental run, Orient = Chamber orientation, Vol = Urine concentration. Bold indicates $p < 0.05$, $n = 12$.

	SS	DF	MS	F	P
Intercept	711072.8	1	711072.8	28364.70	<0.000001
TREAT	3857.3	3	1285.8	51.29	<0.000001
Error	1103.0	44	25.1		
RUN	1.8	1	1.8	0.09	0.768644
RUN*TREAT	20.8	3	6.9	0.34	0.793838
Error	886.9	44	20.2		
ORIENT	102.1	1	102.1	8.11	0.006668
ORIENT*TREAT	115.1	3	38.4	3.05	0.038390
Error	553.8	44	12.6		
VOL	29293.0	5	5858.6	584.98	<0.000001
VOL*TREAT	4437.8	15	295.9	29.54	<0.000001
Error	2203.3	220	10.0		
RUN*ORIENT	0.1	1	0.1	0.00	0.956622
RUN*ORIENT*TREAT	7.2	3	2.4	0.11	0.956655
Error	1011.0	44	23.0		
RUN*VOL	28.1	5	5.6	0.44	0.822521
RUN*VOL*TREAT	680.5	15	45.4	3.53	0.000019
Error	2830.3	220	12.9		
ORIENT*VOL	126.3	5	25.3	1.89	0.096593
ORIENT*VOL*TREAT	239.2	15	15.9	1.20	0.276602
Error	2934.8	220	13.3		
RUN*ORIENT*VOL	78.8	5	15.8	1.09	0.368777
RUN*ORIENT*VOL*TREAT	495.0	15	33.0	2.28	0.005242
Error	3190.2	220	14.5		

Figure 20 shows the response of newly-moulted female prawns to the various temperature treatments tested, with a clear decline in response to nominally increasingly degradative treatments. Also, Table 20 shows that the treated samples were all significantly different from controls (freshwater only) and from one another. Figures 21 and 22 illustrate the effects of run number and chamber orientation respectively on response. Although experimental runs are similar there is a slower response seen in orientation 2.

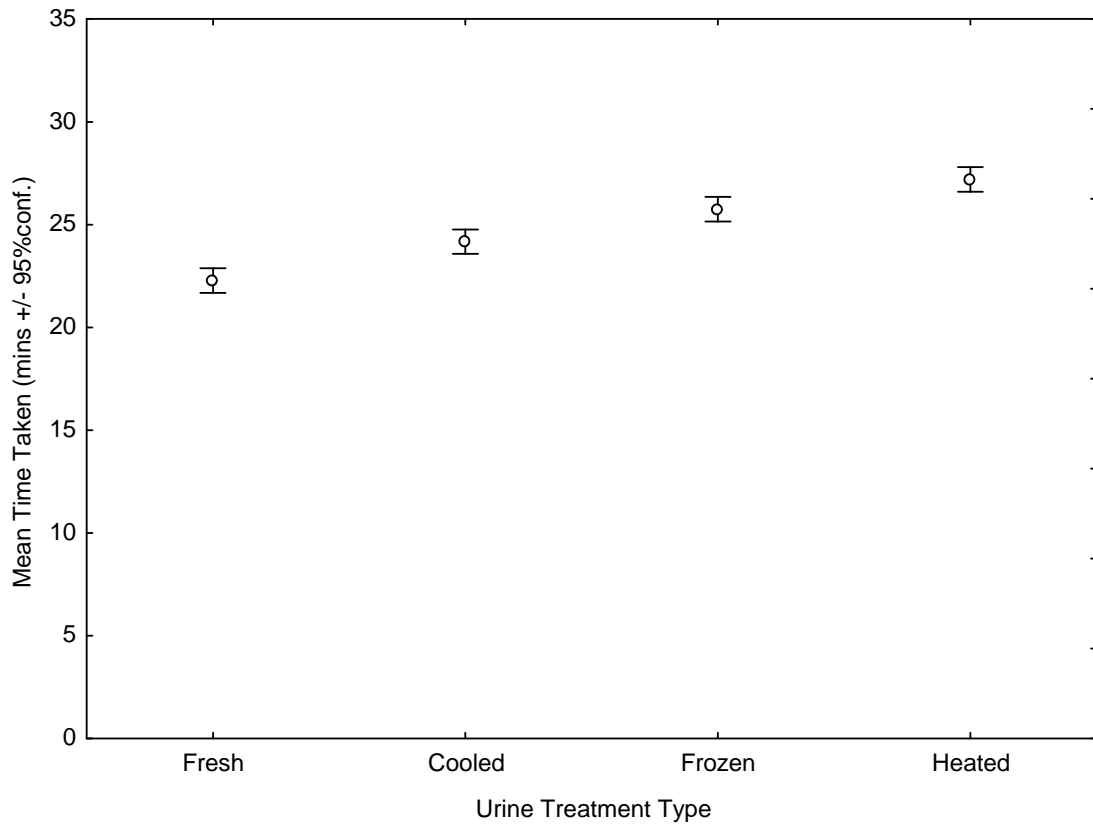


Figure 20: Graph of mean time taken to reach target by newly-moulted females exposed to male blue claw urine treated in a number of ways.

Table 21: Tukey HSD *post-hoc* test for overall time taken by newly-moulted females to reach target exposed to fresh BC male urine. Test for differences in responses to urine treatment. Bold indicates $p < 0.05$.

Urine Treatment	Fresh	Cooled	Frozen	Heated
Fresh				
Cooled	0.000400			
Frozen	0.000169	0.002680		
Heated	0.000169	0.000169	0.006216	

Table 22: The mean of sexual response time (minutes) of newly-moulted females *M. rosenbergii* to BC male urine treated with different temperature treatment.

Urine Treatment	Fresh	Cooled	Frozen	Heated
Mean	22.274	24.167	25.743	27.194

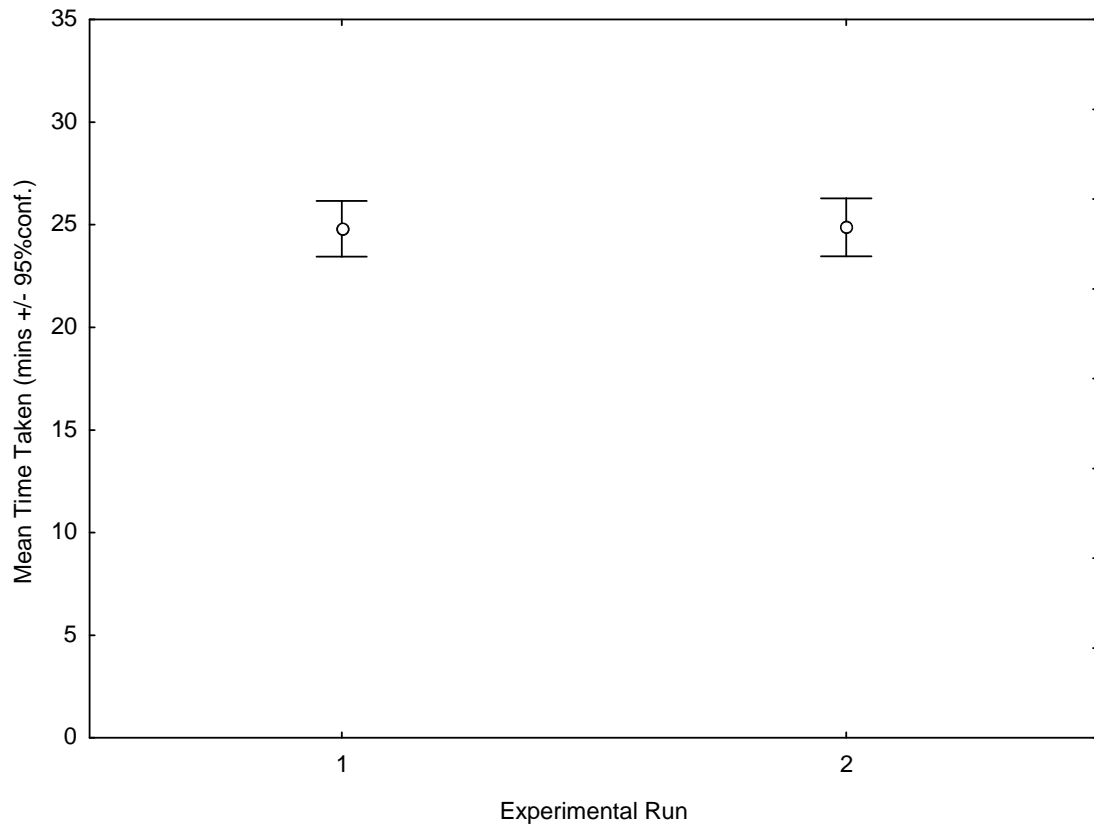


Figure 21: Graph of mean time taken by newly-moulted females to reach target exposed to male BC urine in successive experimental runs.

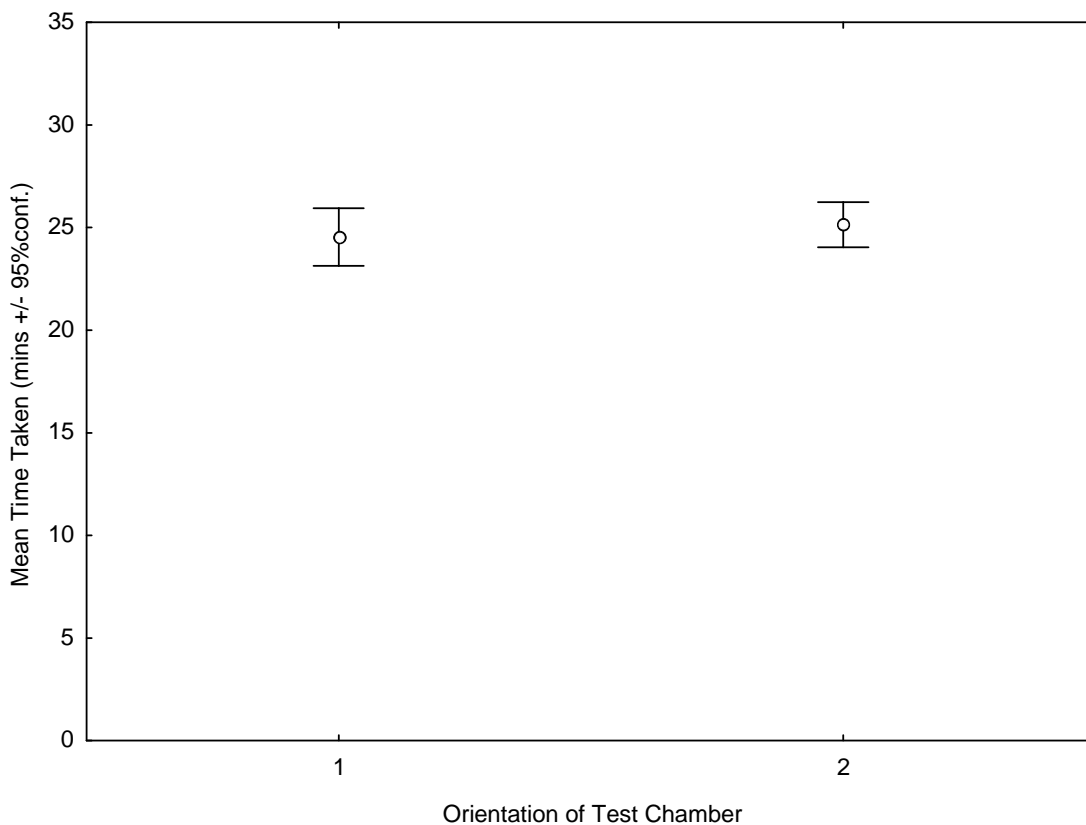


Figure 22: Graph of mean time taken by newly-moulted females to reach target exposed to male blue claw urine with two test chamber orientations.

Table 21 shows a Tukey HSD test for differences between responses to the tested fresh urine concentrations (see also Figure 23). In this experiment, with the larger concentrations analysed overall, all added concentration more than $1.0 \mu\text{l l}^{-1}$ were seen to elicit significantly more rapid responses than controls. All added concentration more than $1.0 \mu\text{l l}^{-1}$ elicited faster responses than lower concentrations except for $2.0 \mu\text{l l}^{-1}$ and $3.0 \mu\text{l l}^{-1}$ which gave similar responses. A Dunnett's test (Table 21) supports the significant difference of all responses to concentrations more than $1.0 \mu\text{l l}^{-1}$ from those seen to controls.

Table 23: Tukey HSD *post-hoc* test for overall time taken by newly-moulted females to reach target exposed to fresh BC male urine. Test for differences in responses to urine concentration. Bold indicates $p < 0.05$, $n = 12$.

Urine Volume	Control	1.0 $\mu\text{l l}^{-1}$	2.0 $\mu\text{l l}^{-1}$	3.0 $\mu\text{l l}^{-1}$	5.0 $\mu\text{l l}^{-1}$	10.0 $\mu\text{l l}^{-1}$
1.0 $\mu\text{l l}^{-1}$	0.980970					
2.0 $\mu\text{l l}^{-1}$	0.016397	0.121797				
3.0 $\mu\text{l l}^{-1}$	0.000021	0.000040	0.191503			
5.0 $\mu\text{l l}^{-1}$	0.000020	0.000020	0.000020	0.000020		
10.0 $\mu\text{l l}^{-1}$	0.000020	0.000020	0.000020	0.000020	0.000020	

Table 24: *Post-hoc* Dunnett's test against control for overall time taken by newly-moulted females to reach target exposed to fresh male blue claw urine. Significance denotes that time taken to reach target is less than control. Test for differences in responses to urine concentration. Bold indicates $p < 0.05$.

Urine volume	Control
	29.651 (average time, min)
1.0 $\mu\text{l l}^{-1}$	0.550221
2.0 $\mu\text{l l}^{-1}$	0.003419
3.0 $\mu\text{l l}^{-1}$	0.000021
5.0 $\mu\text{l l}^{-1}$	0.000021
10.0 $\mu\text{l l}^{-1}$	0.000021

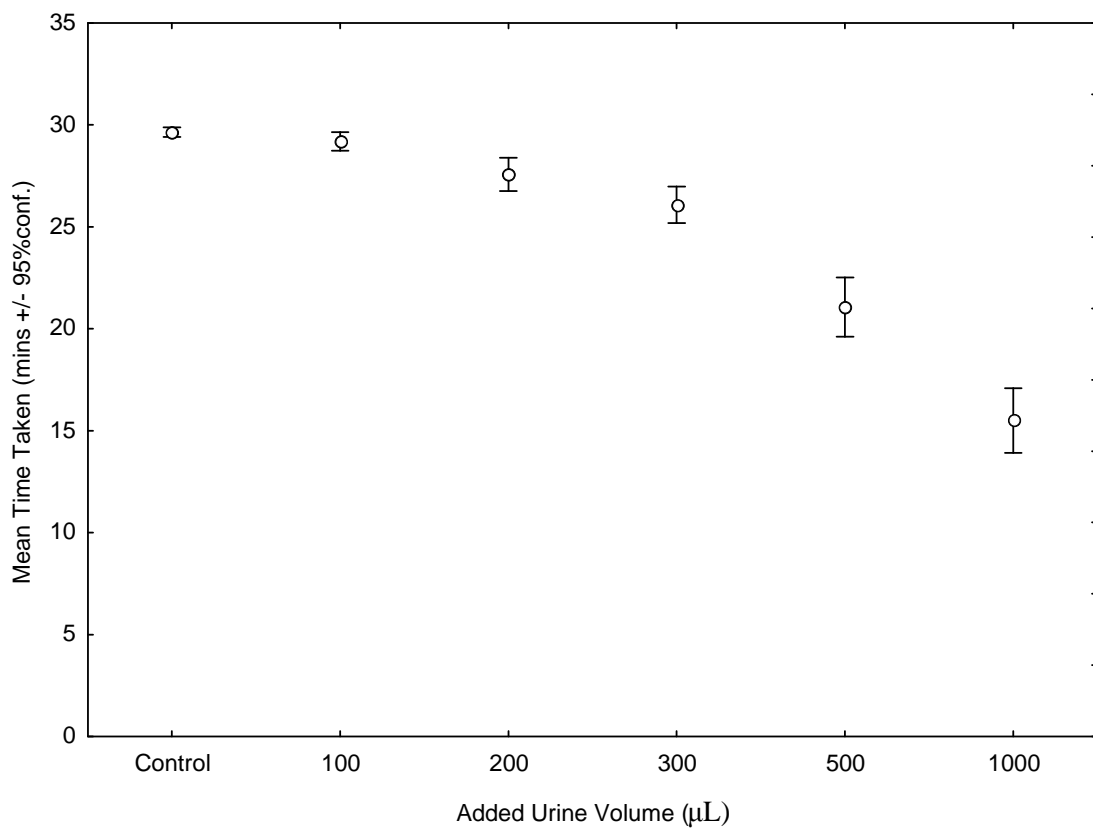


Figure 23: Graph of mean time taken by newly-moulted females to reach target exposed to male blue claw urine with different urine concentrations.

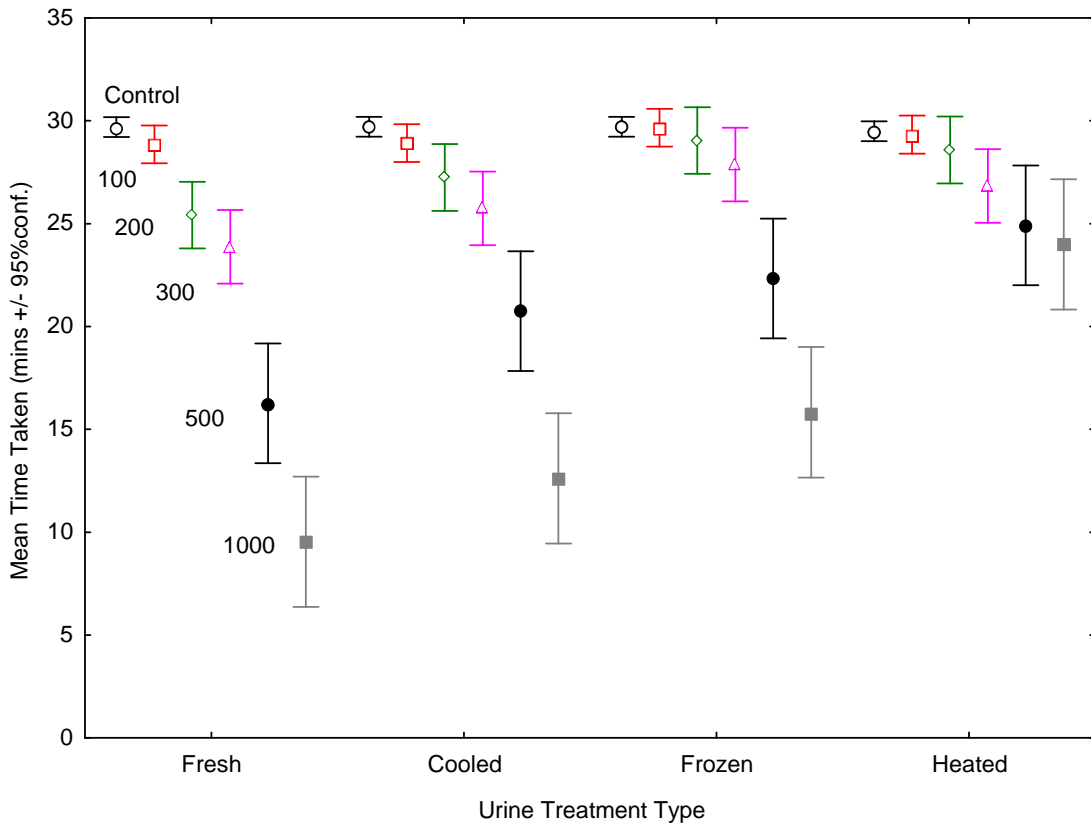


Figure 24: Graph of interactions in mean time taken by newly-moulted females to reach target exposed to male blue claw urine treated with different treatments and with different concentrations ($\mu\text{l l}^{-1}$) of urine.

The significant interactive effect of treatment and urine concentration are shown in Figure 24. Unsurprisingly the effect of degradative temperature treatments is greater where there is a higher initial concentration eliciting a more rapid response from females. Figure 25 shows the significant interactions on female response time of urine concentrations, temperature-treatment and test chamber orientation. It can be seen that there is a marked effect on chamber orientation at the highest concentration ($10.0 \mu\text{l l}^{-1}$) with responses in orientation 2 being slower. This effect is less pronounced / more arbitrary at lower concentrations.

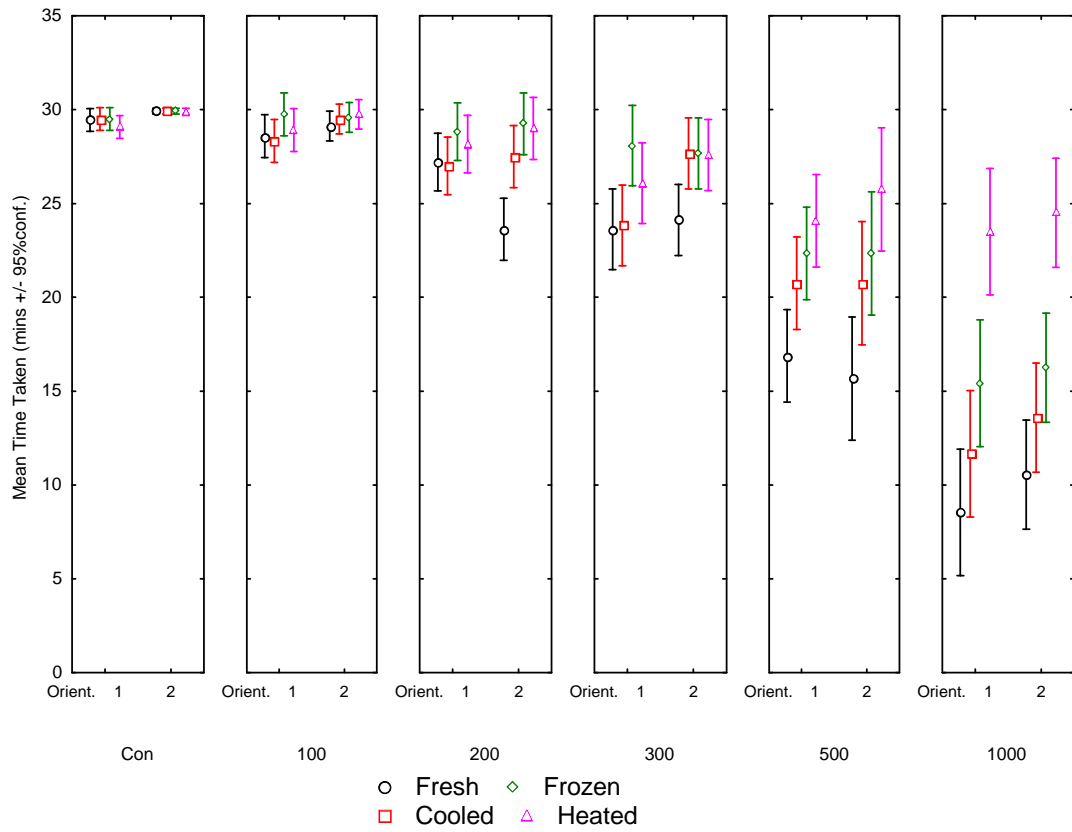


Figure 25: Graph of interactions in mean time taken by newly-moulted females to reach target exposed to BC male urine added at different concentrations, with different temperature treatments and with two test chamber orientations.

4.7 Discussion

Several decades of studies have suggested that urine is one of the major putative sources of pheromone in a number of aquatic crustacean decapod species, including *P. sanguinolentus* (Ryan, 1966), *H. americanus* (McLeese *et al.*, 1977), *C. maenas* (Eales, 1974) and *C. sapidus* (Gleeson, 1980). The present study re-examined previous findings using a behavioural bioassay to compare the responses of sexually receptive newly-moulted female to urine derived from BC male freshwater prawns. The results of these experiments clearly demonstrated positive female behavioural responses which are sexually and temporally specific to BC male freshwater prawn urine.

The findings of this study indicate the existence of important information carried and transmitted through chemicals in the urine of *M. rosenbergii*, which play an important role in the determination and progression of reproductive and dominance status. Observations in this study showed that newly-moulted female *M. rosenbergii* were attracted to BC male urine. This study clearly indicates the important role of urine as a carrying medium for chemical cues rich in information for reproductive recognition and attraction behaviours in *M. rosenbergii*. The presence of sex pheromone in BC male urine is thus well established. These findings coincide with those found in previous studies of some other aquatic crustacean species such as the lobster *H. americanus* (Atema, 1986; Cowan, 1991; Atema, 1995; Bushmann and Atema, 1997; Karavanich and Atema, 1998a), spiny lobsters *J. edwardsii* (Raethke *et al.*, 2004) and *P. argus* (Horner *et al.*, 2006), shore crab *C. maenas* (Bamber and Naylor, 1996a; 1997), *I. dorsettensis* (Jones and Hartnoll, 1997), *T. cheiragonus* (Kamio *et al.*, 2003; 2005), crayfish *O. virilis* (Hazlett, 1985), *P. leniusculus* (Stebbing *et al.*, 2003) *P. clarkii*

(Figler *et al.*, 2005), *O. rusticus* (Simon and Moore, 2007), rotifer *Brachionus plicatilis* (Rico-Martínez and Snell, 1997) and snapping shrimp *A. heterochelis* (Rahman *et al.*, 2001).

Results obtained in the recent study do, however, show some differences to those reported in some other works which showed that some other crustacean species are limited in their ability to detect or respond to other conspecifics through chemically mediated signals alone. Although males increased urine output in response to females, Bushmann and Atema (2000) reported that when urine from male lobster, *H. americanus* was collected and injected into a bioassay system in the presence of a catheterised male, this restored female approach, whilst introducing urine alone did not induce approaching behaviour. However, when they blocked males' urine nephropore, females significantly reduced approaching behaviour towards the male. The latter study did not indicate how much urine dose was injected into the bioassay system for that particular experiment.

4.7.1 Fresh urine concentration

In aquatic crustaceans, urine released by an individual can elicit a positive sexual response from receiving individual *M. rosenbergii*, however, the minimum amount of urine that can invoke a positive sexual response remains to be determined. Thus, if the amount of urine released is restricted into the surrounded environment or controlled, would a receptive prawn respond positively to a given amount of urine, and if so, how long would the response take?

Observations made in this study showed that newly-moulted female *M. rosenbergii* responded positively to fresh urine obtained from BC males at all the concentrations tested. However, the speed of sexual response varies and depends directly upon the urine dose injected into the bioassay tank system. Results obtained here show that the sexual response time of females was elevated with reduction of the BC male fresh urine concentration in a nearly linear profile. In other words, the response seen to fresh urine is directly proportional to the urine concentration released into the bioassay system tank. For instance, at low fresh urine concentrations ($0.1 \mu\text{l l}^{-1}$, $1.0 \mu\text{l l}^{-1}$ and $2.0 \mu\text{l l}^{-1}$), female response time was significantly longer than at the $3.0 \mu\text{l l}^{-1}$, $5.0 \mu\text{l l}^{-1}$ and $10.0 \mu\text{l l}^{-1}$ concentrations.

Atema and Cowan, (1986) observed a positive sexual response in American lobster *H. americanus*, when they compared the seawater control with only one volume, 500 μl of urine. At the same volume 500 μl , similar findings were recorded in the present results. Testing only one urine volume, as reported by Atema and Cowan, (1986), is not adequate to obtain a wider profile of sexual responses. Comparing several levels of urine dose is likely to give a better understanding the effects of urine dose on sexual responses. A report of the minimum volume that could cause positive responses is also lacking in the Atema and Cowan (1986) study. In the current study, although slight sexual responses by newly-moulted female *M. rosenbergii* were noticed at the three fresh urine concentrations $0.1 \mu\text{l l}^{-1}$, $1.0 \mu\text{l l}^{-1}$ and $2.0 \mu\text{l l}^{-1}$, statistical analysis indicates no significant different ($p>0.05$) between these three concentrations. This may indicate that these three particular concentrations are not sufficient to elicit attraction behaviour in newly-moulted females. The Atema and Cowan (1986b) study did not indicate the

time scale of responses, a further important parameter in measuring response. Later, several relative studies on *H. americanus* were conducted by Bushmann and Atema (1994, 1997, 2000), however, none of them were testing the urine concentration parameter on the sexual response behaviour. Thus, further research on different urine volumes in other crustaceans may help explanation of the characteristics and the potent effect of urine volume on sexual behavioural attraction.

Although when $10.0 \mu\text{l l}^{-1}$ of fresh urine were injected into the bioassay system tank, this resulted in the fastest sexual response, $3.0 \mu\text{l l}^{-1}$ was found to be the minimum capable of eliciting a significant ($p < 0.05$) sexual response. Thus, it is possible to suggest that $3.0 \mu\text{l l}^{-1}$ of fresh urine is an adequate concentration to invoke a significant sexual attraction under laboratory conditions. The critical urine concentration of BC males which can elicit a significant sexual attraction of newly-moulted females *M. rosenbergii* is therefore likely to lie between $2.0 \mu\text{l l}^{-1}$ and $3.0 \mu\text{l l}^{-1}$. Final determination of this threshold parameter requires further investigation. Although, the pheromone component quality is important to elicit sexual attraction between male and female prawn, however, a suggestion proposed by Bushmann and Atema (2000) is in contrast to the findings of this study who reported that sexual attraction behaviour between male and female *H. americanus* is qualitative rather than quantitative dependent.

The effects of the experimental tank run, tank orientation and urine concentration on the sexual behaviour of *M. rosenbergii* were tested as shown in Figures 21 and 22. Amongst these three factors, only urine volume had a significant ($p < 0.05$) effect on the sexual behaviour of the newly-moulted female prawn.

4.7.2 Urine temperature treatment

Different temperature-treated BC male urine types were used to test the friability of active components (pheromones) and the effect of degradation upon newly-moulted female sexual behaviour. Results of this experiment showed that temperature treatments reduce the efficiency of urine in terms of sexual attraction of newly-moulted female *M. rosenbergii*. When temperature-treatments were applied to fresh urine collected from BC male prawns, different results were obtained than those obtained previously (in this study) for the fresh urine trials. Amongst the temperature treatments, however, newly-moulted females were slowest in sexual response when they were tested with heated urine.

Significantly, ($p < 0.05$) this experiment showed that newly-moulted females responded to fresh urine faster than to any of the other temperature-treated urine types (cooled, frozen and heated). It can be suggested that temperature therefore affects the capacity of BC male urine to attract newly-moulted females. This suggests that the key chemicals causing sexual attraction behaviour are either degraded or totally destroyed by temperature treatments. These kinds of compounds which are sensitive to temperature could be suggested to be organic chemicals such as protein or carbohydrate or both.

4.7.3 Cooled urine treatment

Cooled-treated (4°C) urine of BC male prawns is significantly different ($p < 0.05$) from the fresh urine. The overall pattern for cooled-treated urine was following similar pattern of fresh urine concentrations in which the sexual responses increase with increasing the injected doses. However, the sexual attraction reactions of newly-

moulted female towards cooled urine concentrations were slower than the fresh urine concentrations. The slow responses by the newly-moulted female may indicate that some of pheromone components in the cool-treated urine were either partly changed or degraded. In comparison between cooled urine type, female showed no significant difference ($p>0.05$) in their responses towards $1.0 \mu\text{l l}^{-1}$ concentration compared to $2.0 \mu\text{l l}^{-1}$ of cooled urine. At concentration of $3.0 \mu\text{l l}^{-1}$ and higher of cooled urine treatment, newly-moulted females showed significantly ($p<0.05$) positive higher responses than towards $1.0 \mu\text{l l}^{-1}$ and $2.0 \mu\text{l l}^{-1}$. Meanwhile, there is no significant difference ($p>0.05$) in sexual response of newly-moulted female between $5.0 \mu\text{l l}^{-1}$ and $10.0 \mu\text{l l}^{-1}$. This probably indicates that $5.0 \mu\text{l l}^{-1}$ is adequate enough to elicit sexual behaviour attraction and any bigger concentration will not make any significant change in the female's attraction behaviour. Results of this study suggest that urine contents were partly degraded in cooling treatment. Thus, the low sexual attraction behaviour of newly-moulted females *M. rosenbergii* towards cooled urine is a clear indication that storing fresh urine even just at low temperature (4°C) had affected its quality and efficiency with time in reproductive communication.

4.7.4 Frozen urine

As shown in the results of this study, sexual responses increased with increasing urine concentrations regardless of temperature treatment type. Obviously, frozen urine is less effective in sexual attraction for newly-moulted female than both the fresh and cooled urine within corresponding concentrations. Results of recent study showed that newly-moulted female *M. rosenbergii* had no significant difference ($p>0.05$) of their sexual response toward concentrations lower than $5.0 \mu\text{l l}^{-1}$ (3.0 , 2.0 and $1.0 \mu\text{l l}^{-1}$) of frozen urine. This indicates clearly that freezing urine at -70°C had partly degraded the

efficiency of pheromone. Relatively, newly-moulted females responded to the cooled urine faster than the frozen urine, so, it can be suggested that freezing treatment (-70°C) to fresh urine is causing more degradation of pheromone than cooling treatment (4°C).

Previous works conducted by Horner *et al.* (2006) who examined chemically mediated sheltering behaviour in *P. argus*, and use it to examine the source and specificity of the aggregation signal. They reported that low concentration 0.01 $\mu\text{l l}^{-1}$ of frozen (-20°C) could be detected by lobster *P. argus*, while in the current study, very low response of *M. rosenbergii* was observed to a concentration of 0.1 $\mu\text{l l}^{-1}$ of frozen urine. Differences in behavioural responses seen with respect to the present study are probably attributable to the differences in experimental design, and perhaps to the quality and the quantity of the collected urine. For example, these authors did not determine (quantify) what was the least significant amount of urine that could elicit a positive response. Also, the manner in which urine was collected, pooled and treated could influence its odour quality, which in turn could affect the behavioural responses of the spiny lobsters. Furthermore, since their experiment was about studying the sexual responses of spiny lobster, the experimental lobster individuals used in their study were sub-adults (young adults) which may have not reached a sexual maturation stage capable of producing sex pheromone. Horner *et al.* (2006) also failed to indicate whether they cleaned the flume bioassay tank after each trial such that the residues from previous trials might affect the following one. Since Horner *et al.* (2006) used artificial (unfrozen) seawater from the flume bioassay tank as the control stimulus, their interpretation might have been more useful if collected urine as well as frozen urine had also employed.

Although there are some differences in the experimental conditions between Bamber and Naylor's 1997 study and this study, however, their findings are broadly in agreement with the results obtained in this study in which sexual behavioural responses increased with increasing urine volumes. Using -20°C frozen urine, they reported that 26 out of 29 of male shore crabs, *C. maenas* showed positive sexual responses to a urine collected from pre-moult female at dilution of $100\ \mu\text{l}$ in one litre of filtered seawater ($100\ \mu\text{l l}^{-1}$) while only 4 out of 29 males showed positive sexual responses to $0.1\ \mu\text{l l}^{-1}$. For frozen urine (-70°C) in this study, significant positive sexual responses of newly-moulted females *M. rosenbergii* were found only at $5.0\ \mu\text{l l}^{-1}$ and $10.0\ \mu\text{l l}^{-1}$ of urine concentrations, which are far away from what have been reported by Bamber and Naylor (1997) who used urine concentrations of 0.1, 1.0, 10 and $100\ \mu\text{l l}^{-1}$.

Differences between Bamber and Naylor (1997) findings and the results obtained in the present study are probably due to the involvement of several factors including experimental conditions, level of sensitivity to pheromone and chemical content of urine. One of the experimental conditions differing in Bamber and Naylor's 1997 study is the procedure for collecting and storing urine. They collected urine at the end of the day, pooled it and then stored it in a freezer at -20°C , while in this study fresh urine was collected and stored immediately in a freezer at -70°C . Freezing urine at -70°C may preserve sex pheromone more efficiently than -20°C and this may affect attraction behaviour. Second, it may be related to the level of sensitivity to the pheromone. Shore crab *C. maenas* males may have responded to the very low urine concentration of pre-moult female because probably they have higher chemo-receptive sensors than newly-moulted female *M. rosenbergii*. Also, the sex pheromone substances of pre-moult

female shore crabs may differ in their attractive efficiency from BC male sex pheromones. It has been reported that male but not female crustaceans control (increase and decrease) their urine release according to the opponents individual status (Breithaupt *et al.*, 1999; Breithaupt and Atema, 2000; Zulandt-Schneider and Moore, 2000). It is worth mentioning that urine of freshwater Crustacea such as *M. rosenbergii* will be more dilute compared to those of marine Crustacea species. Accordingly, such difference between these two environmental media could be a considerable factor in producing varying concentrations of pheromone. Thus, although females' urine might have an important function in reproductive behaviour the urine volume factor may be less important to sexual attractive behaviour than males' because urine released from males often shows not only reproductive status but also position in the social hierarchy (Ra'anan and Cohen, 1985). Finally, behavioural responses may be affected by study time since the experimental observation time period in the Bamber and Naylor (1997) study was 60 minutes which is exactly double the length applied in this study. It should be noted finally that the volume of 100 μ l in Bamber and Naylor (1997) study is surely not 100% pure urine, as compared to this study, but rather a diluted solution of urine in seawater.

4.7.5 Heated urine

Results of this study for heated urine shows a similar pattern to the other temperature treated urine. Although the time taken for female responses became slightly faster with increasing heated urine concentrations, however, these responses were not statistically significant ($p > 0.05$). Studies testing the effects of high temperature treatment (heated or/and boiled) urine on attractive properties in other animal taxa is limited. In insects, Ono (1992) reported that the components of sex pheromone of the potato tuberworm

moth, *Phthorimaea operculella*, were influenced by the rearing temperature in which sex pheromone percentage dropped as the rearing temperature was raised. Another laboratory experiment to study the effect of temperature and humidity on sex pheromone, but not urine, production in corn earworm *H. zea* was conducted by Raina (2003). Raina's study tested the effect of three temperature treatments (15, 25 and 35°C) on sex pheromone volume in the female corn earworm and it was observed that both cool and warm (15 and 35°C) temperature treatments reduced sex pheromone concentration in females. This differences, however, is more likely to be due to differences in pheromone production than to differences in pheromone degradation. It is irrational to compare both of Ono (1992) and Raina (2003) studies, or any similar, with the current findings of *M. rosenbergii* simply because both were applied on terrestrial insects which are different than the aquatic crustacean. However, one of the most important point to be highlighted out of these two studies is that pheromone has critical sensitivity to the surrounded temperature. As shown in the urine of BC males heating urine disintegrated the key substance(s) of pheromone involved in sexual attraction.

Studies testing high temperature treatment (heated or/and boiled) of urine for aquatic crustacean seems to be largely absent from the literature. Nagabhushanam *et al.* (1989) focused on the effect of conditioned water carrying sex pheromone on the female's ovary maturation but not on sexual response of the freshwater prawn *M. kistnensis*. In their study they tested boiled conditioned water but not urine. The content of conditioned water is likely to comprise more than one excreted compound of which urine is only one component. Nagabhushanam *et al.* (1989) showed clearly that temperature treatment of conditioned water, *e.g.* boiling degraded responses. This

probably indicates that the effective substance in causing ovary maturation was changed or damaged when boiled. In comparison, high temperature treatment of urine as carried out in this study had effects consistent with those reported by Nagabhushanam *et al.* (1989). Thus, exposing pheromone to high temperature, whether heating or boiling, probably degrades the chemical cues associated with the reproductive behaviour of freshwater prawn and this in turn effect the chemical properties of the attractive active component of urine. Further investigation of the differential effects heat treatments on urine or pheromone can determine more about optimal temperature tolerance of the active factors and about the chemical nature of the active component.

Chapter 5 Antennal gland: Morphology and function

5.1 Antennal gland

The antennal gland (AG) also known sometimes as the green or maxillary gland, has been studied and described as an excretory organ in several crustacean species such as the crayfish: *Cambarus sp.* (Anderson and Beams, 1956), *Austropotamobius pallipes* (Riegel, 1963, 1965, 1966), *Orconectes virilis* (Riegel, 1963; Kirschner and Wagner, 1965; Shivers and Chauvin, 1977), *O. limosus* (Riegel, 1965), *Astacus astacus* (Vogt, 2002), *Procambarus blandingi* (Peterson and Loizzi, 1973, 1974), *P. clarkii* (Schaffner and Rodewald, 1978; Wheatly *et al.*, 2004), *P. leniusculus* (Kirschner and Wagner, 1965; Fuller *et al.*, 1989), and *A. leptodactylus* (Khodabandeh *et al.*, 2005ab).

The AG has also been described in other crustacean species: the blue crab, *C. sapidus* (Johnson, 1980); the brachyuran ghost crab, *Ocypode albicans* (Flemister, 1959), the fiddler crab, *Uca mordax* (Schmidt-Nielsen *et al.*, 1968), *Cancer borealis* (Holliday and Miller, 1984), the horseshoe crab, *Limulus polyphemus* (Briggs and Moss, 1997), the lobster, *H. gammarus* (Burger, 1957; Benhke *et al.*, 1990; Bushmann and Atema, 1996b; Dove, 2005; Khodabandeh *et al.*, 2005c), the spiny lobster, *J. edwardsi* (Binns and Peterson, 1969), the freshwater cherry or red shrimp, *Neocaridina denticulata* (Ueno *et al.*, 1997), *P. monodon* (Lin *et al.*, 2000) and the amphipod, *Corophium curispinum* (Taylor and Harris, 1986).

Other literature has suggested that the tegumental gland is an important excretory organ in which removes excess, unnecessary or dangerous materials from an organism as in crustaceans as have been reported in some other decapods such as *Derocheilocaris*

typica (Crustacea, Mystacocarida) (Elofsson and Hessler, 2005; Hessler and Elofsson, 2007). It has been proposed that the maxillary gland in some crustacean species as a principal excretory organs such as in brine shrimp, *Artemia salina* (Crustacea, Branchipoda) (Tyson, 1969); isopods, *Asellus aquaticus* (Crustacea, Malacostraca) (Walter and Wägele, 1990); Branchypoda, *Hutchinsoniella macracantha* (Crustacea, Cephalocarida) (Hessler and Elofsson, 1991) and *Anthrobathynella stammeri*, (Syncarida, Malacostraca) (Steenken and Schminke, 1996).

It has been demonstrated that the excretory system in crustaceans *i.e.* the AGs is derived from the segmental nephridial of ancestral taxa (Johnson, 1980). Since the AG in crustacean is responsible for excreting urine which is believed to carry the sex pheromone used in reproductive behaviour, this chapter will principally focus on this organ. The AG in crustacean is an essential excretory organ whose main function is to eliminate metabolic wastes, regulate haemolymph and control osmotic regulation (Vogt, 2002). To maintain osmotic homeostasis of the haemolymph hypertonic, the superfluous water must be withdrawn from the haemolymph and discharged from the body without losing electrolytes (Vogt, 2002). Moreover, Vogt (2002) stated that there were experimental evidence that the AG of crayfish is also involved in excretion of organic xenobiotics and heavy metals. Decapod crustaceans have a bilateral pair of AGs which are located at the base of each antenna in the anterior ventral region of the cephalothorax. Generally speaking, each of these glands functionally is equivalent to the kidneys of mammals, connecting with two primitive ducts leading to excretory pores open to the external environment. Each AG has a single opening (nephropore) which is located on the underside of the coxae of the flagellum antenna (Vogt, 2002).

5.1.1 Antennal gland morphology

Several previous studies in crustaceans have provided different morphological descriptions of the AG. Depending on whether the crustaceans are marine or freshwater species, the literature shows similarities and differences in describing the morphological and functional aspects of the AGs. Some authors reported that the AG is composed of three regions while others found four. In some crustaceans, especially freshwater crayfish species, a nephridial canal joins the labyrinth tubule with the bladder cavity while this kind of tubule is missing in marine crustacean species such as crabs (Schmidt-Nielsen *et al.*, 1968; Peterson and Loizzi, 1974).

Among those studies which proposed that the AGs of crustaceans possess three regions are those of Goodrich (1946); Parry (1955); Peterson and Loizzi (1973, 1974); Holliday and Miller (1984); Fuller *et al.*, (1989); Benhke *et al.* (1990); Arkarajamon (1991); Nakamura and Nishigaki (1991); Rinderhagen *et al.* (2000) and Xiaoyun *et al.* (2003). Other studies which suggested that AGs consisted of four regions include those of Johnson (1980); Vogt (2002); Wheatley *et al.* (2004) and Shuang-lin and Nai-cheng (2007). It seems that a nephridial tubule is the principal feature which distinguishes between the two groups. This particular portion of the AG in the freshwater species acts as a salt-reabsorbing mechanism. A brief coverage of the morphological and functional aspects of the AGs for both two groups is provided in this chapter.

Early on, Goodrich (1946) reviewed the previous literature concerning the AGs of different crustacean species and published from the year 1895 till 1946. He reported the excretory system of crustaceans as having three distinct parts (a) a proximal hollow end

sac (coelomosac) opening into (b) a narrow excretory canal merging to (c) a distal duct (labyrinth) of ectodermal origin, terminating in the opening pore on the base of the second antenna. Also, a brief review is given by Panikkar (1941) of descriptions of the morphology of the excretory organs of palaemonid prawns. He showed that each AG comprised an end-sac, tubular labyrinth and bladder. The end sac lies at the base of the antenna and has a small compact coelomosac and its wall is considerably folded. The inner layer is complicated and lined by large epithelial cells which have clear nuclei and granular cytoplasm, whereas, the outer layer contains a connective tissue with blood spaces. Second, the labyrinth which consists of an anastomosing network of tubules is formed from a single layer of epithelial cells leads to the bladder. The bladder is the last part of the AG. At the end of bladder there is a short duct leading to a nephropore, which is located at the base of the antennary peduncle on a small papilla.

Later, Peterson and Loizzi (1973, 1974) demonstrated that the main regions of the freshwater crayfish, *P. blandingi*, AG are the coelomosac, labyrinth and nephridial canal. They gave a brief description from a morphological and functional point of view of these three main regions. The coelomosac comprises substantial cells joined together by several junction areas but they are connected to the basal lamina by pedicels. Within the cytoplasm, there are various vacuoles and vesicles of different sizes. According to the staining tendency and morphological characteristics, Peterson and Loizzi (1973, 1974) demonstrated that the labyrinth of *P. blandingi* can be distinguished into two distinct parts. This particular region of the AG has cuboidal and columnar cells which have a brush border, long and narrow intercellular spaces, basal plasmalemmal (cell membrane) invaginations and typical cytoplasmic components. These authors suggested

that in *P. blandingi* the adjacent areas of epithelium may have nuclei that are either homogeneous or granular in both secreting and non-secreting epithelia. The nephridial canal region does not have a brush border and consists of cells that have extensive basal invaginations associated with elongated mitochondria. The proximal cells in this region are small and filled with mitochondria throughout. The cytoplasm contains several vesicles and vacuoles, free ribosomes, dense bodies, diffuse glycogen, and some rough endoplasmic reticulum. Compared to the proximal cells, the distal cells are large, less compact, and cuboidal to columnar in shape. The cytoplasm of the distal cells is similar to that of the proximal cells, but it differs in respect to the basal invaginations which are even larger and more extensive. In both proximal and distal region cells, it is suggested that active solute reabsorption occurs, probably against an osmotic gradient.

Holliday and Miller (1984) demonstrated that the labyrinth and bladder in crayfish are connected with a tubule. Using standard techniques for scanning electron microscopy and ultrasonic micro-dissection, Fuller *et al.* (1989), reported that the AG of the crayfish *P. leniusculus*, similarly consists of three major parts. First, the coelomosac which contains elongated cells (podocytes) with microvilli at the apical surface. Also, they stated that a smooth basal lamina was found to contour the blood space that supplies haemolymph to the coelomosac. Second, the labyrinth consists of tall columnar cells with apical microvilli. Third, the nephron tubule which consists of two distinct areas: a proximal region that has flattened cells with extensive intercellular fusions, and a distal region of separated dome-shaped cells.

For the lobster, *H. americanus*, Behnke *et al.* (1990) also found that the AG has three main regions, the coelomosac, the labyrinth and the bladder. In a physiological comparison between the AG of crustaceans and the kidney of mammals, Behnke *et al.* (1990) reported that both the AG and the kidneys work alike but that the AG is structurally less complex. The mammalian kidney is composed of a multitude of single nephron units each contributing to overall filtration and reabsorption, while the AG in crustaceans does not have this degree of complexity (Behnke *et al.*, 1990). Also, the fine structure of epithelial tissues in the coelomosac is analogous to the podocyte tissues in the Bowman's capsule of vertebrates (Schmidt-Nielsen *et al.*, 1968; Tyson, 1968; Peterson and Loizzi, 1974).

For shrimp, Nakamura and Nishigaki (1991) demonstrated that the AG of the kuruma prawn, *P. japonicus*, is composed of a coelomosac, labyrinth and urinary bladder. Both the coelomosac and the urinary bladder showed a complex variety of vesicles. Later, Xiaoyun *et al.* (2003) examined the AG under light and electron microscopy. They showed that the AG of the shrimp, *P. chinensis*, consists of a coelomosac, labyrinth and nephridial canal. The coelomosac wall is composed of podocytes and a basement membrane. The podocytes are connected with the basement membrane at the basal side and project towards the lumen at the apical side. The blood carried by the blood vessels and small arteries is filtered across the basement membrane of the coelomosac and thus moves from wall of the small arteries into the coelomosac to form primary urine. The labyrinth columnar cells display microvilli and cell membrane invaginations. Finally, the nephridial tubules come after the labyrinth in which the basal cell membranes are

invaginated. Nevertheless, the lateral cell membranes of the epithelium of the nephridial tubules are deeper and larger than those of the epithelial cells in the labyrinth.

Other authors suggested that the AG of crustaceans comprise four regions. For the blue crab, Johnson (1980) suggested that each AG consists of four regions, the apical coelomosac (end sac), labyrinth (excretory or renal tubule), nephridial canal and the bladder. He demonstrated that the coelomosac is of mesodermal tissue origin while the labyrinth, nephridial canal and the bladder are all of ectodermal origin. He demonstrated that the coelomosac tubule is formed from a single layer of podocyte epithelial cells, lacks connective tissues, vacuolated toward the lumen and that the apical margins of these cells are thin and tenuous. The nuclei of the podocyte cells are small, granular and dense and basally located while the nucleoli are not clear. The cells of the coelomosac luminal surface, however, contain various bodies with different sizes where the large ones extruded cytoplasmic inclusions. The two opposing epithelia of the coelomosac and the labyrinth are joined across the haemal space irregularly and sometimes appear adjacent. Cells of the labyrinth epithelium have apical pinocytotic vesicles, lysosome-like dense bodies on the basal side and abundant mitochondria, especially basally. He demonstrated that cells in the epithelial tissue of the bladder are very similar to those of the labyrinth and appear to have similar shape whether in secretory or non-secretory condition. Compared to the bladder, however, he reported that variation in the intensity of secretory activity and in the height of epithelial cells tends to be less than in the labyrinth. In addition, the basal portion of the cytoplasm has some indistinct fibrils than the labyrinth and there is less glycogen present than in the labyrinth.

Johnson (1980) reported that the appearance of epithelial tissues in the labyrinth tubule of the blue crab differed according to their physiological state; thus when in a non-secretory phase, cells are varied in their shape from cubical to low columnar. Also, nuclei contained a distinct nucleolus located either centrally or apically and were homogeneous or granular. The cytoplasm in the labyrinth cells appears to have two main zones, the basal and the apical zones. The basal zone contains threadlike fibres “fibrillar” and extends from the basal cell membrane and ends at the middle of the cytoplasm and contains many mitochondria. The apical zone has an abundance of distinct long microvilli derived from the apical cell membrane and free on their terminated ends. When the crab is in a secretory condition, the cells of the labyrinth are columnar, the apical cytoplasm is very vacuolated, the apical margins sometimes may have tenuous (vague) appearance and the nuclei are mostly close to the cell basal membrane (Johnson, 1980). The lumen of the labyrinth showed different widths, sometimes being narrow and sometimes large and wide (Johnson, 1980) but when the gland is in a non-secretory phase, the lumen appears narrow.

Vogt (2002) described the AG of the crayfish *A. astacus* (Figure 26) as consisting of four distinct regions, the coelomosac, labyrinth, nephridial tubule and the bladder along with nephropore. The coelomosac is suggested to be the site of the primary urine formation after ultrafiltration of the haemolymph. The coelomosac possess of a central chamber and various radiated and short tubules which are tightly interdigitated together with haemal sinus (vesicles). The very basic coelomosac epithelium is composed a single layer of podocyte cells. The podocyte cells are sending down dendritic foot processes, the pedicels which intermingled with a thick basal lamina. This basal lamina

separates the urinary space from the haemal space. The spaces between of these pedicels are linked by the slit diaphragm. Usually, the nuclei of podocytes cells are apical with irregular shape and each cell is separated laterally by different size of intercellular spaces which are extending from the haemal space through the urinary spaces to the coelomosac lumen. These spaces however, are essential and acting as a main route of the ultra-filtrate fluids to transfer from the basal to the apical side toward the coelomosac lumen.

The labyrinth is tubular and is lined by epithelial tissues comprising either cuboidal and/or columnar cells which have a brush border of microvilli along the apical cell membrane. The lumen of labyrinth is divided into numerous of small chambers forming as extensive surface of blebs. It has been suggested that one of the functions of the labyrinth is reabsorption of substances to be retained in the haemolymph (Vogt, 2002). The nephridial tubule occupies the largest portion of the AG and has two distinct regions: a proximal bladder which is located next to the labyrinth and has more flattened cells and a distal region near the bladder which has cuboidal cells. The epithelial cell layers of the nephridial tubule lack a microvillar or brush border. The bladder consists of epithelial cells that are flat and non-secretory and lacks muscle cells. Its main function is to hold urine and then discharge it out through the nephropore.

Wheatly *et al.* (2004) found that the AG of the crayfish, *P. clarkia*, consists of a coelomosac leading to a labyrinth, nephridial canal and the bladder. Each of these parts has a certain function, the coelomosac for urine ultrafiltration, the nephridial canal is for reabsorption and the bladder is for urine storage. The distal nephridial tubule is a region

not present in marine crustacean species which produces isoionic urine. Also, calcium re-absorption occurs in the nephridial tubule/labyrinth (Wheatly *et al.*, 2004).

In other crayfish species, using scanning electron microscopy, Khodabandeh *et al.* (2005a) briefly described the main parts of the AG in the crayfish, *A. leptodactylus*, which showed that the AG comprised four main parts. The coelomosac has a single layer of epithelial cells forming a central tubular chamber with smaller tubules. The podocyte cells in the coelomosac appear to have distinctive pedicels on the basal lamina while at the apical side cells are set in an adjacent order and they are intercellularly attached together. The apical cells form a honey-comb like small haemolymph cavity which may increase surface contact between the coelomosac cells and the haemolymph. These cells possess round or oval nuclei with several lobes, vacuoles, plenty of intercellular dense bodies and vesicles.

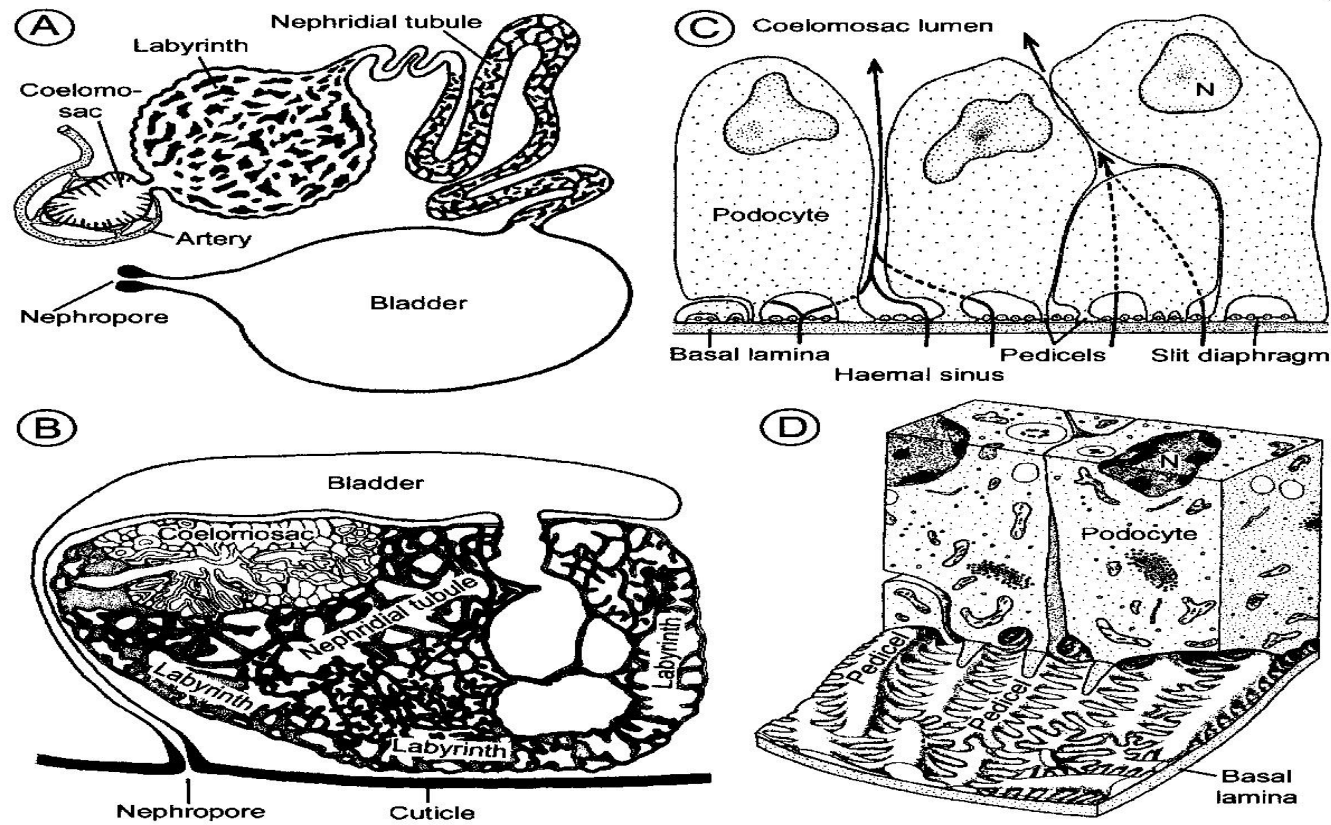


Figure 26: Diagrams of the antennal gland (AG). (A) Simplified scheme of unraveled AG of *Orconectes limosus*. (B) Section through AG of *Procambarus clarkii* showing segments of organ in natural position. (C) Coelomosac epithelium of podocytes of *O. limosus* composed of haemolymph filtrate. N: cell nucleus. (D) Block diagram of podocytes with pedicels. From Vogt (2002) In: Holdich (2002), A, C, D after Kümmel, 1964; B after Maluf, 1939)

The coelomosac region leads to the labyrinth. Khodabandeh *et al.* (2005a) reported that the haemolymph in the crayfish *A. leptodactylus* supplied by vessels to the labyrinth cells is bathing (surrounding) these cells. The cells of the labyrinth can be categorised into two distinct types; I and II. Labyrinth I cells have apical microvilli and the cells are cubical in morphology with a basal nucleus. In these cells, the large apical cytoplasmic extrudes into the haemolymph contents toward the lumen and often the extruded cytoplasm has an influence in distributing the apical microvilli. Many round mitochondria and vesicles are present at the apical end of these cells, while in the basal zone of the cells there is an irregular cell membrane infoldings with new mitochondria. The labyrinth II cells, however, are columnar and possess dense bodies, basal membrane infoldings, septate junctions and apical microvilli. The microvilli in the brush border are interspersed with several bodies containing dense uniform cytoplasm. The mitochondria are round and evenly distributed through the cell and the nucleus is usually centrally located.

The nephridial tubule is the third region which comes immediately after the labyrinth. There are two sub-regions of tubule that can be observed with both of them lacking microvilli. The cells of the proximal tubule are cuboids with a central nucleus and many apical intercellular junctions. The apical cytoplasm possesses few vesicles and few organelles. Some of the apical membrane bulges into the lumen and sometimes the cytoplasmic extrusions are present. Deep irregular basal infoldings at the basal side are closely associated with mitochondria. In the distal tubule, however, the cells are columnar with a large nucleus and the cytoplasm possesses abundant apical vesicles and vacuoles, Golgi complexes and dense bodies. The cells of the distal tubule are located

close to and below the coelomosac and the cytoplasm are rich with a network of vacuoles. The luminal area in this part is less than in the proximal part. In tubule cells, the basal infoldings extend deeper than those in the labyrinth cells.

The fourth region of the AG is the bladder in which the cells are dome-like lined by a single layer of epithelium and covered by connective tissue, with microvilli absent at their apical surface. The cells are separated into two regions by the nucleus. The cells possess clear large vacuoles and vesicles and the apical part bulges into the lumen. In some cells, part of the apical region can be seen detached into the bladder lumen. The basal region of the cell, however, appears to be dense and have deep basal infoldings with abundant of mitochondria and light vacuoles. Both mitochondria and infoldings are much more abundant in the bladder cells than any other region of the AG.

Dove (2005) has described the AG of the lobster, *H. americanus*, as consisting of four major regions, coelomosac, tubular labyrinth, bladder and excretory pore. The coelomosac is considered as a primary filtration zone, which is analogous to the glomerular kidney cortex in mammals. The labyrinth is considered as a secondary modification zone which is analogous to the medullary kidney.

5.1.2 Function

The antennal glands of crustaceans are believed to have a considerable role in the osmoregulation process whether in marine or freshwater species. Crustaceans such as crayfish who spend most of their life in freshwater can manage the water and ion

concentrations inside their bodies by adapting three physiological mechanisms and Khodabandeh *et al.* (2005a) summarized those mechanisms as: (1) a low permeability of ions and prevent water invasion; (2) an active uptake of ions from food and by the epithelial branchial chambers ion transporting tissues (Riegel, 1963, 1966; Mantel and Farmer, 1983; Wheatly and Gannon, 1995); (3) the production of hypotonic urine via excretory AG.

The structure of cells in the AG regions suggests their function (Mantel and Farmer, 1983). The process of excretion through the AG involves both ultrafiltration (passive) and secretion (active). Ultrafiltration in the crustacean AG has been studied and reviewed by several authors. To a greater or lesser extent, the literature shows evidence that the AG functions as an osmotic and ionic regulator in both freshwater and marine crustaceans. Osmoregulation is one of the main functions of the AG in crustaceans in which regulate the water and ion contents between the internal body and the surrounding water medium. Also, the AGs may work as a tool to excrete the metabolic waste products outside the animal's body. However, each region of the AG has a particular task which they work in an integrated fashion.

It has been reported earlier that podocyte tissues in crustaceans play a principal role in ultrafiltration and secretion of the primary urine and may also act in reabsorbing proteins (Peterson and Loizzi, 1974). These authors suggested that the main function of the coelomosac is as a filtration mechanism with reabsorption of materials such as protein occurring subsequently from the filtrate and secretion of other substances into the lumen. They reported that there are two distinguishable sub-regions, the labyrinth I

that has cells suggested to move fluid isototically across the epithelium and the labyrinth II, which is probably involved in isotonic transport and appears to be more active in the endocytic uptake and intracellular digestion of large molecules such as proteins.

The tubule connecting the labyrinth and the bladder in crayfish has been reported to be a possible site for salt absorption and production of dilute urine (Riegel, 1963, 1965). This was also reported later in the crab, *C. borealis* (Holliday and Miller, 1984). Kirschner and Wagner (1965) mentioned that the coelomosac in crayfish *Pacifastacus sp.* and *O. virilis* is the site of formation of a primary ultra-filtrate. Furthermore, they suggested that 90–95% of water in the blood (haemolymph) entering the coelomosac is filtered and passes into the more distal regions of the coelomosac. Mantel and Farmer (1983) also reported that the AG in marine decapods is involved not only in the control of haemolymph volume but is also involved in hypo-regulation of magnesium and sulphate in the haemolymph, excretion of organic substances and the reabsorption of fluid, sugars and amino acids from the primary urine filtrate. Khodabandeh *et al.* (2005b) stated that the ultrafiltration process in the AG of crayfish *A. leptodactylus* occurs across the wall of the coelomosac and that the filtrate moves through several parts of the efferent duct where it is modified through secretion and absorption processes to produce dilute urine.

Lin *et al.* (2000) conducted an experiment testing the ion and body fluid volume regulation of cannulated *P. monodon* reared in different salinities (5, 25 and 45‰) of seawater. They demonstrated that the urinary production rate of *P. monodon* decreased

as the water salinity increased and they suggested that the AG not only regulates the fluid volume but also the concentration of sodium and potassium in their body. They concluded that sodium and potassium concentrations are regulated by the antennal gland after an unexpected change in seawater salinity levels. Also, Xiaoyun *et al.* (2003) described the AG function of *P. chinensis*. They suggested that the podocyte cells in the coelomosac play a role in absorbing the large molecules from the primary urine, and in intra-cellular digestion and detoxification. The tubules in the labyrinth are able to reabsorb water, ions and large molecules, regulating the osmotic pressure and actively eliminating waste products. The epithelial cells of the nephridial tubules can further absorb water and ions from the lumen of the nephridial tubules. Also, it may play an important role in the formation of the highly osmotic urine.

5.2 Urine dynamics in the AG

In crustaceans, before urine becomes a waste product, it goes through a complicated process where a variety of modifications occur inside the AG. The literature approaches urine dynamics from several different points of view. In crayfish *A. pallipes* and *O. virilis*, Riegel (1963) found that the osmotic pressure of the urine in all parts of the AG except the coelomosac is significantly hypo-osmotic compared to the haemolymph and that the osmotic pressure of the urine declined most sharply between the distal portion of the distal tubule and the bladder. However, the urine in the coelomosac can be either hypo or hyper-osmotic to the blood (Riegel, 1963). He reported that there is no direct evidence of arterial pressures in the AG which is high enough to effect filtration under hydrostatic pressure. Riegel (1963, 1965) concluded that the arterial pressure in the AG

of crayfish must therefore come from sources other than the haemocoel pressure but he did not specify what source is.

5.2.1 Production of urine

Synthesis of urine goes through a complex of processes starting with ultrafiltration through secretion and ending with reabsorption and probably also passive diffusion where each of these processes occurs in different regions of the AG. In the crab, *C. borealis*, Holliday and Miller (1984) demonstrated that the haemolymph is carried by the antennal artery through the coelomosac in the crustacean antennal gland where it is ultra-filtered under arterial pressure across the wall of coelomosac into its lumen. Then, the filtered fluid leaves the coelomosac and passes to the labyrinth via an opening in its anterior wall. The labyrinth contains an epithelium where urine is separated from the haemolymph across the tissue.

Vogt (2002) showed a brief demonstration of urine production in crustaceans, especially in the freshwater crayfish *A. astacus* in which urine production is only slightly different from that of marine crustaceans. The ultrafiltration is carried out in the coelomosac region, the secretion mainly occurring in the epithelia of the coelomosac and labyrinth and the reabsorption performed by the labyrinth and nephridial tubule. Although the bladder occupies the largest region in the AG and plays a role of urine storage, it may have a further role in dilution of urine. He reported that between 60 and 70% of the primary urine is reabsorbed on its way from the labyrinth to the bladder and ends nearly iso-osmotic to the haemolymph. Wheatly and Gannon (1995) reported that 90 - 95% of the electrolytes are reabsorbed in the AG. The distal nephridial tubule is the primarily

site of ion reabsorption (Vogt, 2002). No or very little of glucose is found in the urine and small amounts of amino acids may be found in the urine but their concentration is always much lower than in the haemolymph (Vogt, 2002). This therefore indicates that the vast majority of sugar is reabsorbed in the AG (somewhere between the coelomosac and the bladder) but the specific site of this process has not been identified. Due to the presence of glycogen in the labyrinth epithelium, Vogt (2002) pointed out that this particular part of the AG is probably involved in sugar reabsorption.

Reabsorption in the AG, particularly of electrolytes and potential metabolites such as protein and glucose may be involved in urine production. Binns and Peterson (1969) found that urine nitrogen contributes 11.6% of total nitrogen loss from the AG of spiny lobster, *J. edwardsi*, with ammonia, urea and amino compounds representing 21.2% of total urine nitrogen. Because most of the nitrogen excreted is non-urinary, they concluded that the AG of the spiny lobster, *J. edwardsi*, is not important as far as total nitrogen loss is concerned.

The moult status of crustaceans may have an influence on the precipitation level of some ions in the AG. In a histochemical study, Rogers and Wheatly (1997) investigated calcium precipitation in the AG during the moulting cycle of the crayfish *P. clarkii*. They found that Ca²⁺ precipitation in coelomosac tissues does not vary significantly, while in the labyrinth and nephridial canal tissues it was significantly more abundant during post-moult than during the inter-moult. They suggested that Ca²⁺ accumulates in re-absorptive tissues during post-moult. Also, they found that distal nephridial canal

tissues are rich in mitochondria suggesting that these mitochondria may serve to maintain intracellular and extracellular Ca^{2+} homeostasis.

5.2.2 Source of sex pheromone

Generally speaking, animal taxa release different types of chemicals from their bodies which are considered as pheromones. These pheromones are usually used for inter and intra-species communication. The organ(s) producing such pheromones vary between groups. In crustaceans, however, the vast majority of research has focused on the importance of urine in communication for agonistic and reproductive activities whereas little attention was given to where the production of such pheromones could be taking place. Thus, for a comprehensive understanding of the role of chemicals in communication, especially during reproductive activities, studying the source of sex pheromones is required.

For crustaceans, pheromones are likely released with the urine (Breithaupt and Atema, 1993; Zulant-Schneider and Moore, 2000), which is corroborated by the fact that urine is released almost exclusively during social interactions (Zulant-Schneider *et al.*, 2001; Breithaupt and Eger, 2002; Bergman *et al.*, 2005). Chemical communication between individual aquatic crustaceans can be caused by either metabolic components transported with urine or / and some other chemical cues released with urine. There is evidence in several species of crustacean that a pheromone is carried with urine but the source of this pheromone is unknown and is still ambiguous. Thus, this implies that these chemical cues consist of either general metabolic products, a unique substance or

mixture, that is produced in the AG and/or in other source (gland) which are then released into the urine (Dunham, 1978).

There are some studies focused on the morphology and the structure of organs producing and release the pheromone (pheromone glands) and the mechanism for producing pheromone in several animal taxa. However, this kind of approach is extremely limited in crustacean species. There have been a few attempts to investigate a sex pheromone gland for crustaceans; however, details of the structure and histology of such glands provoke some arguments. Authors have addressed different approaches to the sex pheromone sources glands.

A number of potential studies on organ(s) that is involved in pheromone production in crustaceans have been suggested. For instance, McLeese *et al.* (1977) concluded that the pheromone may originate in the ovary when they examined chemical and behavioural aspects of the sex attractant of the lobster, *H. americanus*. Jones and Hartnoll (1997) have shown pheromone production in spider crabs to be associated with ripe ovaries. In contrast, Nagabhushanam *et al.* (1989) suggested that the male of the freshwater prawn, *M. kistnensis* is the producer of the sex pheromone and the organs that produce this might be either the vas deferens or the testes.

In addition, other authors have proposed other glands as a pheromone source in some crustacean species such as tegumental, sternal and rosette glands. For example, Bauer (1979) suggested that the tegumental gland is probably involved in tanning newly

formed cuticle along with producing some specific substance related to mating behaviour of the caridean shrimp, *H. paludicola* female.

Early work on the histological and anatomical properties of the “sternal gland” for the freshwater prawn, *P. paucidens*, suggested their being pheromone glands (Kamiguchi, 1972). He suggested that the sternal gland is closely related to the reproductive cycle. Also, he proposed that the sternal gland possesses rosettes cells which surround a central lumen. He found that the sternal glands of *P. paucidens* are located in the inner side of the 3rd, 4th and 5th pereopods and beneath the thoracic sternum between the pair of 5th pereopods. Throughout his study, he observed that the sternal gland consists of roughly 600 – 800 glandular structures (or rosette) forming seven separate groups.

The existence of the rosette gland may vary according to gender or age. Kamiguchi (1972) found that the rosette glands are only found in the mature female’s body and they are totally absent in the male and in immature females (smaller than 7 mm CL) while the first rosette gland detected appeared in about 7.8 mm CL females. However, the fully developed rosette gland was observed in sexually-mature females larger than 10 mm CL. These glands could be visually recognised when the female is in the last hours before moulting. In this case, the glands have a milky white colouration which can be identified through the cuticle, while it is difficult to be distinguished at the end or after the breeding season.

Each of the glandular structures (or rosette) possesses 10–14 cells and each has a spherical nucleus. These rosette cells are arranged in a row of circular shape with one common centre and an outlet duct that extends from the central lumen to the outside through the cuticle. Products of these glands are released via ducts that open directly into the external environment through the cuticle immediately following mating moult. According to the tinctorial properties of their secretory substances and to the histological staining colouration, Kamiguchi (1972) categorised the rosette gland into two types; α - and β -rosette cells, where α -rosettes represent the vast majority of the rosette glands (95%).

The number and the size of the sternal glands vary depending on the prawns' growth and age. Kamiguchi (1972) concluded that growth of the sternal glands is correlated with the gonadal maturation of the prawns. In addition, from their appearance histologically, he noticed that the sternal glands secretion in the female prawns is affected by the moult cycle. For instance, the maximum size of the rosette gland was observed five minutes after moult. Also, from the colouration of the rosette outlet duct stains, he suggested that the secretory product is likely to be actively discharged immediately following the mating moult. However, half an hour after mating moult, the secretory material was found only at the central part of the rosette gland. Two to eight hours post-mating moult, the rosette glands are almost empty of secretory material. Furthermore, he suggested that the rosette glands are able to regenerate secretion material after 3 days of mating moult. So, Kamiguchi (1972) concluded that the timing of rosette gland secretions with the female's mating moult is an indication of using such chemical materials in reproductive purposes to attract males.

Bushmann and Atema (1993; 1996b) examined the histological structure of the nephropore area of the lobster, *H. americanus*. Results of both studies are reviewed here. Their results proposed that the nephropore rosettes vary in size among individuals and contained large masses of rosette glands. In addition, there are some granule cells scattered and attached to the sphincter muscle. Bushmann and Atema (1996b) reported that rosette glands could be found in three main locations of in the lobster is the nephropore, the eyestalks and the pleopods. The rosette glands in the first location the “nephropore“ is more complicated and more organised than the other two. The nephropore rosette glands are found inside cluster cells, in which they are formed into two gland complexes lateral and medial to the ureter. Each of these complexes is attached with one duct where they then join together forming one main duct running along the posterior side of the animal and terminating in the bladder. Also, they noticed rosette chemical materials running in a duct starting from the central lumen and going to either the cuticle of the carapace or the lumen of the ureter. They suggested that the activity of these glands follows the moult cycle in which, at inter-moult, the glands are less developed and look smaller. Results of both studies revealed that individual rosette glands contained either rounded cells with multi secretory granules, or thinner cells with fewer granules. Also, they found that some rosettes were arranged into clusters of different sizes, wrapped in connective tissue. Furthermore, depending on the rosettes location, they reported that these clusters were arranged into two positions, those lateral to the ureter and those medial to the ureter, with both of them having a similar mean size. Each of these rosette cluster complexes are served by one duct and both of these ducts ran posterior and joined to form one common duct terminating in the bladder of the AG. The first are small glands each of which possess a rosette structure which is a cluster of 8 – 12 cells surrounding a central lumen with basal nuclei. Second, a drainage

rosette duct which runs from the central lumen to either the cuticle of the carapace or the lumen of the ureter. Some anterior clusters, however, may release their products via individual ducts directly to the exterior of the body. Using this kind of releasing arrangement, Bushmann and Atema (1996b) suggested that lobsters are able to release rosette secretion products, alone without urine or with the concomitant release of urine. Moreover, they suggested that the rosette gland complexes appear to contain actively secreting cells throughout the year and seems to be active in inter-moult, pre-moult and freshly moulted lobsters. These observations suggested to them that such glands are following the moult cycle and the vesicles contained secretory product. Although the actual function of the rosette gland in crustaceans is not determined yet and since these glands may control the release of chemical cues involved in reproduction, Bushmann and Atema (1996b) concluded that such chemical products are likely to be good candidates to be sex pheromones.

From a 45 minute period of behavioural observation, Bushmann (1999) demonstrated that the blue crab, *C. sapidus*, displayed great variability and flexibility in courtship behaviour which is mainly regulated by both urine and non-urine chemical signals emanating from both males and females. He suggested that chemical signals which may be released from sites other than urine appear to carry sufficient information to elicit a full range of courtship behaviour. Thus, he concluded that courtship between males and females in *C. sapidus* is regulated by multiple sources of chemical signals. Also, he concluded that the release site of non-urine chemical signals is unknown and therefore that it was unknown whether these chemicals are similar to the urine compounds or not.

5.3 Objectives

Since urine plays a significant role in reproductive activities within crustacean species, more detailed focus on urine as a carrier of a complex of chemicals has been conducted. Investigation of the organ producing urine i.e. the antennal gland in crustaceans has, however, received far less attention. This limitation to studies of the crustacean's urine excretion system makes it more difficult to obtain a comprehensive overview of the AG. More detailed research on the morphology and the function of AG could provide a better understanding of this system and therefore a better understanding of its relation to reproductive behaviour. The main objective of this chapter therefore is to focus on the functional morphology of the AG in *M. rosenbergii*. There are, however, some sub-objectives that are addressed in the following study:

- 1) To provide a descriptive function according to all morphology described.
- 2) To ascribe likely function according to the morphology described.
- 3) To place the functional morphology described for the AG of *M. rosenbergii* in context with AGs described for other species of Crustacea.

5.4 Materials and methods

5.4.1 Histology

5.4.2 Tissue samples, preservation and processing

Histological work of the AG tissue samples were conducted as described in the section 2.3 page 69.

5.4.3 Microscopy

Each of the above prepared slides was viewed under a light microscope (Olympus BX51). This particular microscope is provided with a camera (AxioCam MRc, Zeiss) to take the images what is on the microscopic stage. Proper images of slides were photographed and the images were saved at the computer to be printed later on. Also, the TEM was used to investigate the micro details of the AG specimens which are not observable in the light microscope. Sample preparing and the process of the TEM was illustrated in the section 2.4.1 on page 74.

5.4.4 Scale bar

Using a slide of graticule stage which has a scale of 1 mm, different magnification (4, 10, 20 and 40x) images of the scale measures were taken. With the aid of computer software ImageJ V. 1.41, the measurement of the image length was converted from mm into μm units and the scale bar measurements were then attached on the AG image.

5.5 Results

5.5.1 The AG morphology

Cross-section of the cephalothorax region of the *M. rosenbergii*, as presented in the Figure 27 showed that the AG can be found in the ventral side of the prawn's body anterior to the cephalothorax and beside or posterior to the hepatopancreas. However, after removing the AG surgically from ventral locations of both sides of the *M. rosenbergii* prawn's head underneath the antennal base, the morphological shape of AGs showed a massive rounded structure which can be easily seen by naked eyes. However, when these AGs were processed and prepared for the light microscopy, images obtained are showing further morphological details. Under the light microscope each AG showed as rounded body structure as in the Figure 27. Obviously, the AG can be recognised by its location which is in the ventral side of the prawn's body beside or posterior to the hepatopancreas. Each AG comprised different regions in which each has columns cells forming tubule-like structure. The AG displays four well-separated distinct regions: the coelomosac, the labyrinth, the nephridial tubule and the bladder. However, in the Figures 27 and 28 showed the main three distinct regions of the bladder, the labyrinth and the coelomosac.

5.5.2 The coelomosac

Observations under the light microscope revealed that the area of the coelomosac region is relatively smaller compared to the labyrinth and it can be recognised by its location which comes closely attached and posterior to the labyrinth. The coelomosac region, however, has a distinct lumen (duct) or urinary space at the middle part of the region. This duct leads to the labyrinth region. There is a wall located between the coelomosac

and the labyrinth regions (see Figure 28A) indicating that both regions are separated as shown in the Figure 27B.

The cells of coelomosac region are highly vacuolated and located basally close to the cell membrane where nuclei are relatively small elongated or sometimes have irregular shape. Large vacuoles are abundant apically holding various sizes of granular deposits which cause difficulties to recognise the lumen of these kinds of tubules.

5.5.3 The labyrinth

As shown in Figures 27 and 28, the light microscopic micrographs revealed that the AGs of *M. rosenbergii* are located anterior to the cephalothorax and beside or posterior to the hepatopancreas. The area of the labyrinth region is more voluminous and occupying greater bulk of the AG in the *M. rosenbergii*. Also, Figure 27 and Figure 28 showed that the labyrinth located anterior to the coelomosac. This region is full of tubule structures forming a complicated coil-shape. As shown, this region is lack of central lumen or duct. Each tubule has one layer of epithelium cells and the nucleus of these cells can be seen as dark spots. According to the staining (Haematoxylin and Eosin), the tubule density of arrangement and the morphological characters, labyrinth region can be distinguished into two sub-regions, labyrinth-I and labyrinth-II. The staining colouration in the labyrinth-I is slightly darker than the labyrinth-II. The spaces between tubules (haemolymph spaces) are also varying where the labyrinth-I tubules have narrow spaces compared to the labyrinth-II which are relatively wider. The lumen areas (inside the tubule) are also differing in which the labyrinth-I have constricted lumen while the lumen in the labyrinth-II tubules have wider lumen. The internal layer

of the labyrinth tubule is rich with brush border microvilli. Figure 29 (below) demonstrates these differences.

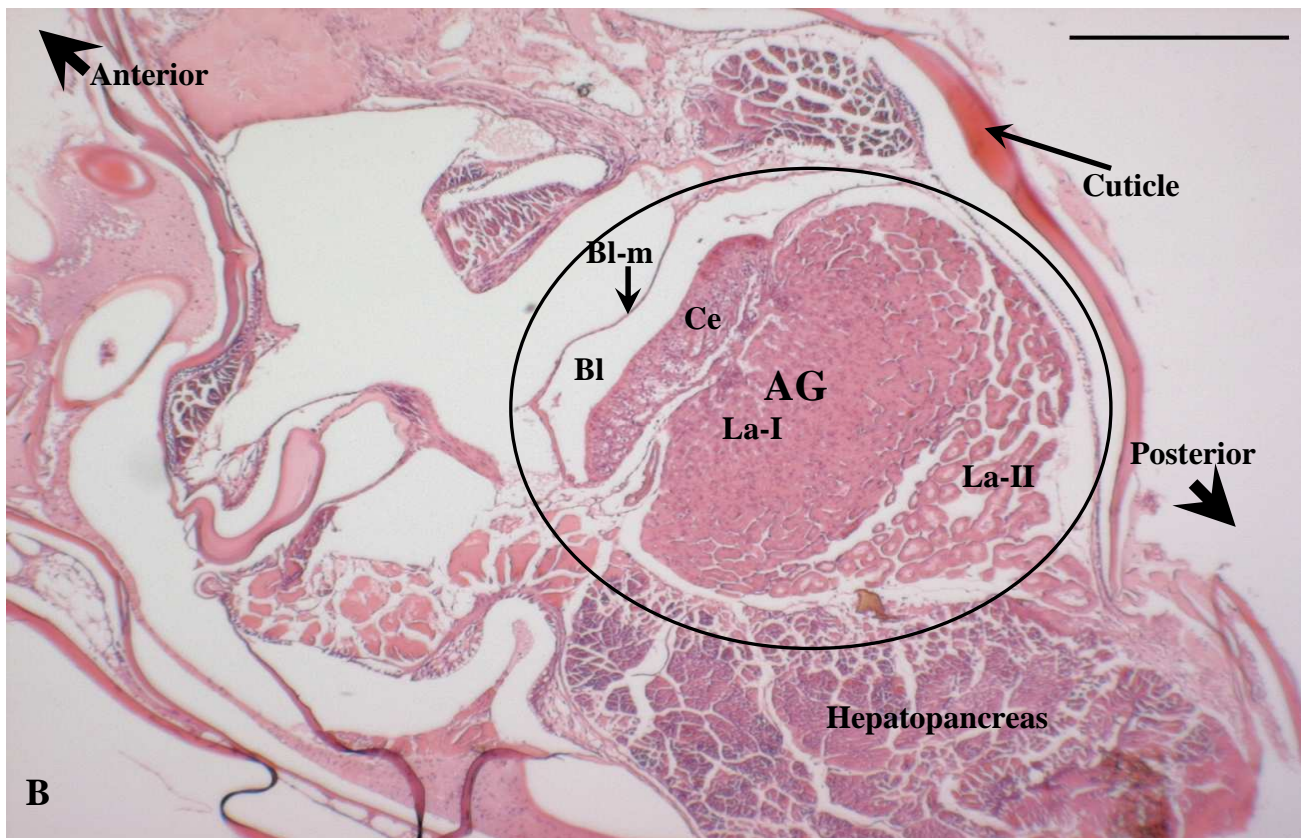
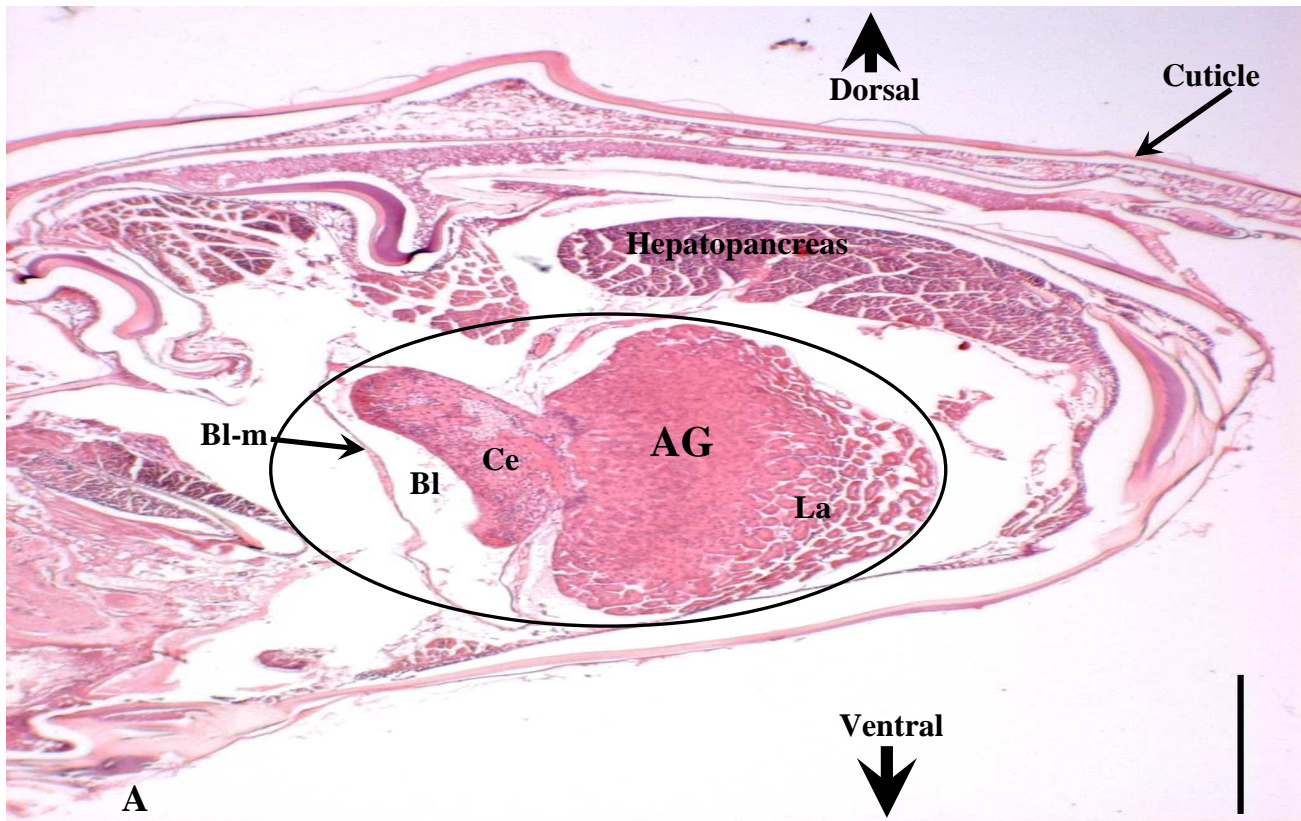


Figure 27: Light micrographs of Haematoxylin and Eosin (H & E) stained section of the internal cross-sectional view of cephalothorax region of two different AGs (A: left and B: right) of the *M. rosenbergii*. Ce= coelomosac, La-I= labyrinth-I, La-II= labyrinth-II, Bl= bladder, Bl-m= bladder membrane. Scale bar = 200 μ m.

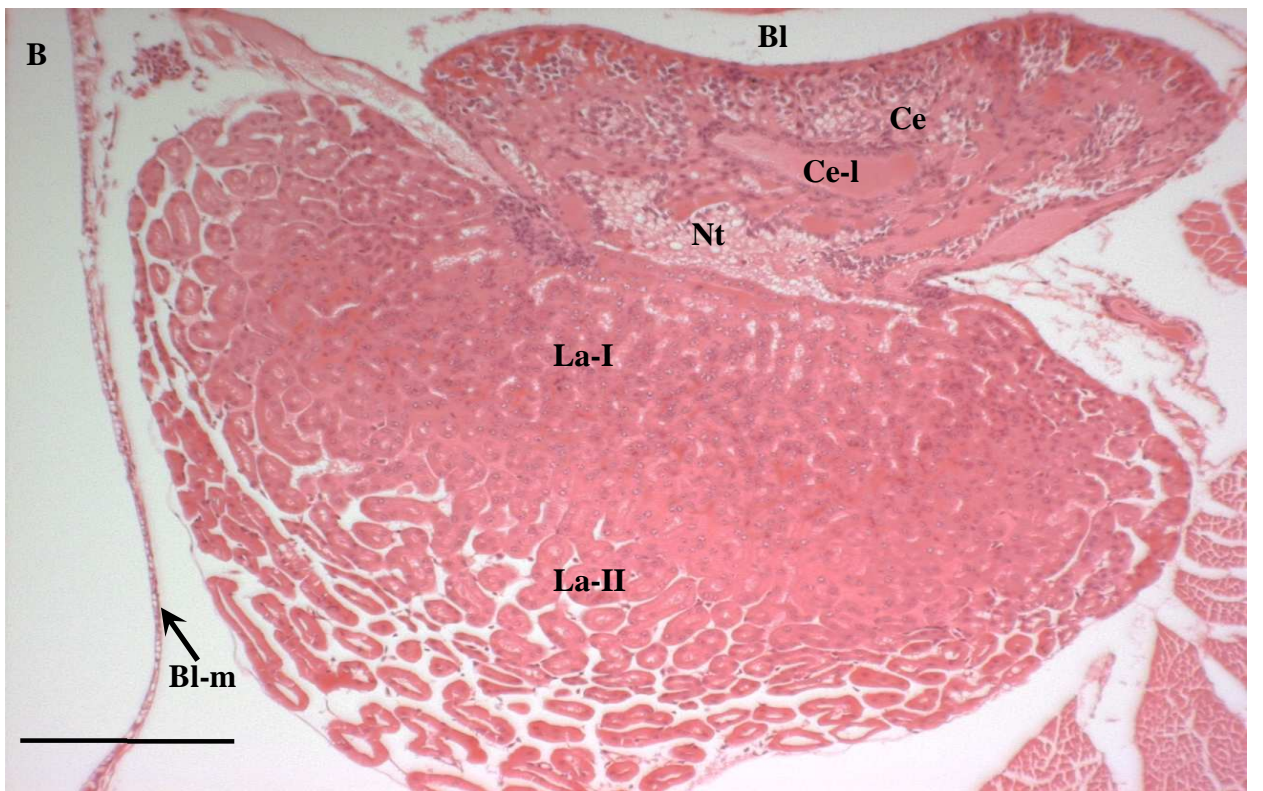
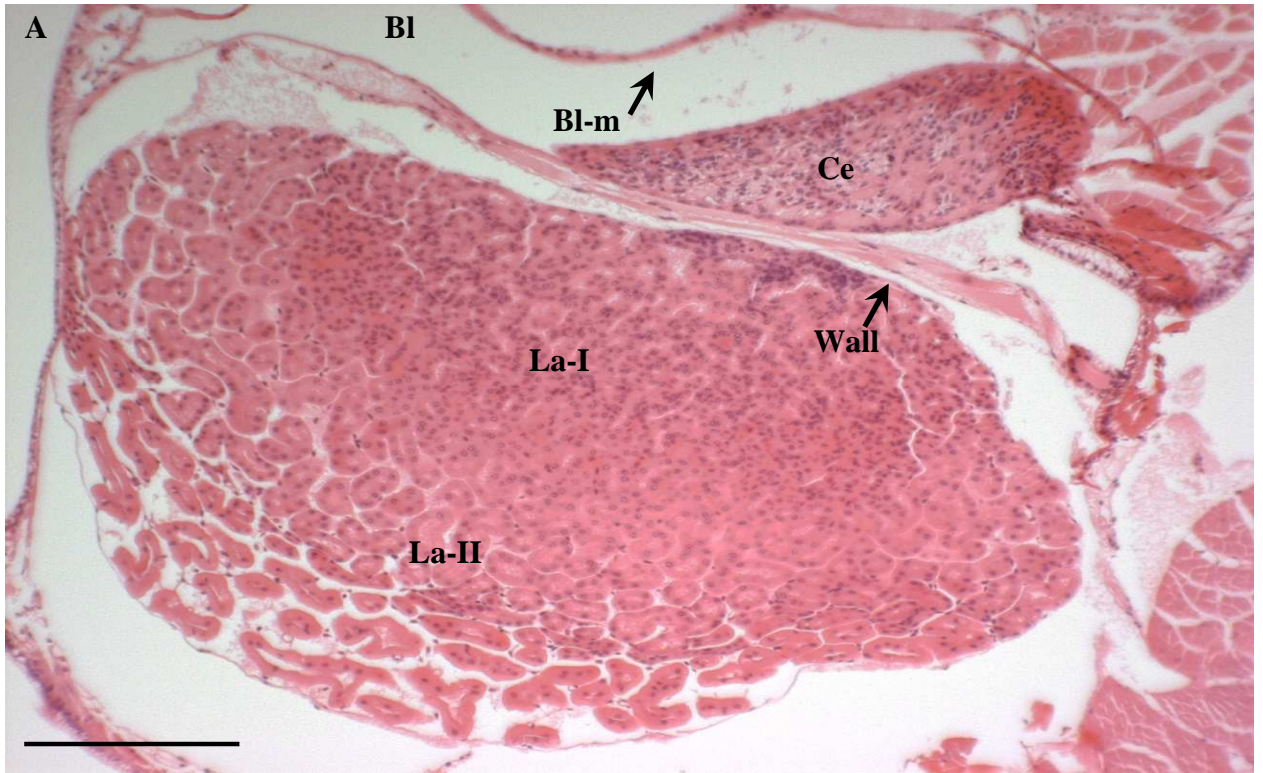


Figure 28: Light micrograph of H & E stained section showing overview structure of two different AGs (A and B) of the *M. rosenbergii*. Bl= bladder, Bl-m= bladder membrane, Ce= coelomosac, Ce-l= coelomosac lumen, La-I= labyrinth-I, La-II= labyrinth-II, Nt= nephridial tubules. Scale bar = 200 μ m.

5.5.4 The bladder

The bladder is the fourth region in the AG. It is a large sac (reservoir) where the lumen is occupying most area of this region. Throughout histological cutting position of the AG section it appears that the bladder is wrapping up the whole AG organ which suggests that it is a large vacuole sac as shown in Figures 27, 28 and 30. However, naturally it is located beside the other regions of the AG. As shown in the Figure 30, the bladder wall has two lines of cell layers, the bladder membrane (basal) and the bladder epithelium (apical) cells. In each of these lines, cells are containing one nucleus. Cells in the apical layer are rounded in their shape and their size is bigger than those in the basal layer which are flattened and cubical. Also, there are plenty of cytoplasmic extrusion vacuoles in the apical cells. Cells in the apical layer have some short and widely distributed microvilli. These two layers are forming a wall which makes it a unique feature compared to the other regions of the AG and then provoke more flexibility to hold and store urine. The bladder liberates urine via the two nephroporse which are located on the last part of the bladder by the aim of nearby muscles or arises of lumen pressure of the AG.

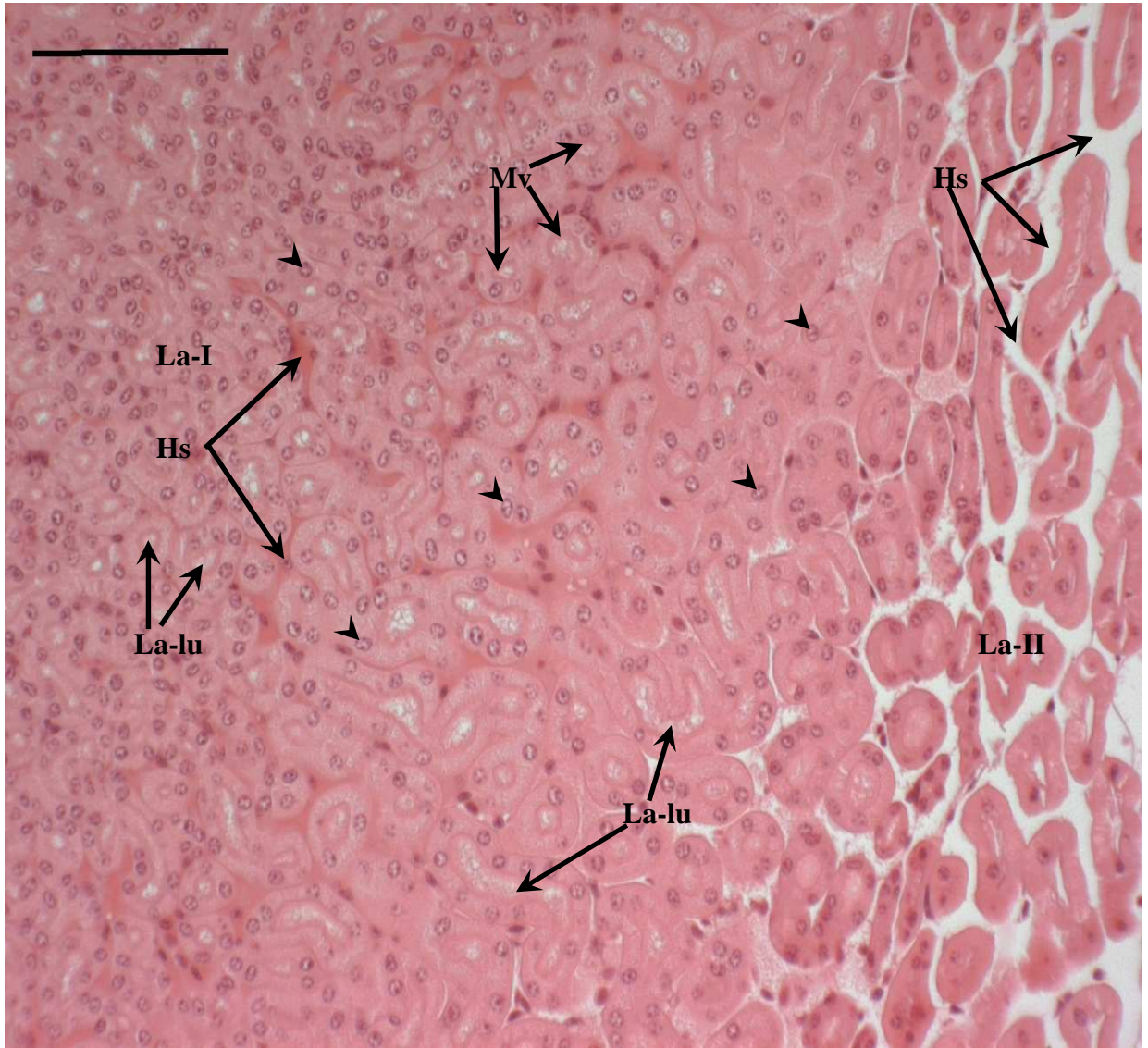


Figure 29: Light micrograph of H & E stained section showing overview structure of two different sub-regions of the labyrinth in the AG of the *M. rosenbergii*. La-I= labyrinth-I, La-II= labyrinth-II, Hs= haemolymph spaces, La-lu= labyrinth lumen, Mv= microvilli border, Nu= nucleus (arrow heads). Scale bar = 200 μ m.



Figure 30: Light micrograph of H & E stained section showing overview of bladder in the AG of the *M. rosenbergii*. BI-l= bladder lumen, BI-a= bladder apical, BI-b= bladder basal, BI-ce= bladder cytoplasmic extrusion, La-II= labyrinth-II, La-m= labyrinth membrane. Scale bar = 250 μ m.

5.5.5 The nephridial tubules

Light microscopy revealed that the nephridial tubules come in thin layers tubule format. The histological cutting in this study showed two different positions appearance of nephridial tubules in the AG, where some times it is shows beside the labyrinth (as showed inside the circled area Figure 31) and some times shows beside the coelomosac (see Figure 33). The nephridial tubules usually appear in a coil net of tubes where the epithelium tissue of these tubules usually has a thin layer of cells which contains nucleus and a dense of basal invagination cell membrane. Also tubules are lacking of microvillus brush border. The structure of the thin layer of nephridial tubules is probably used for pumping ions actively against a concentration.

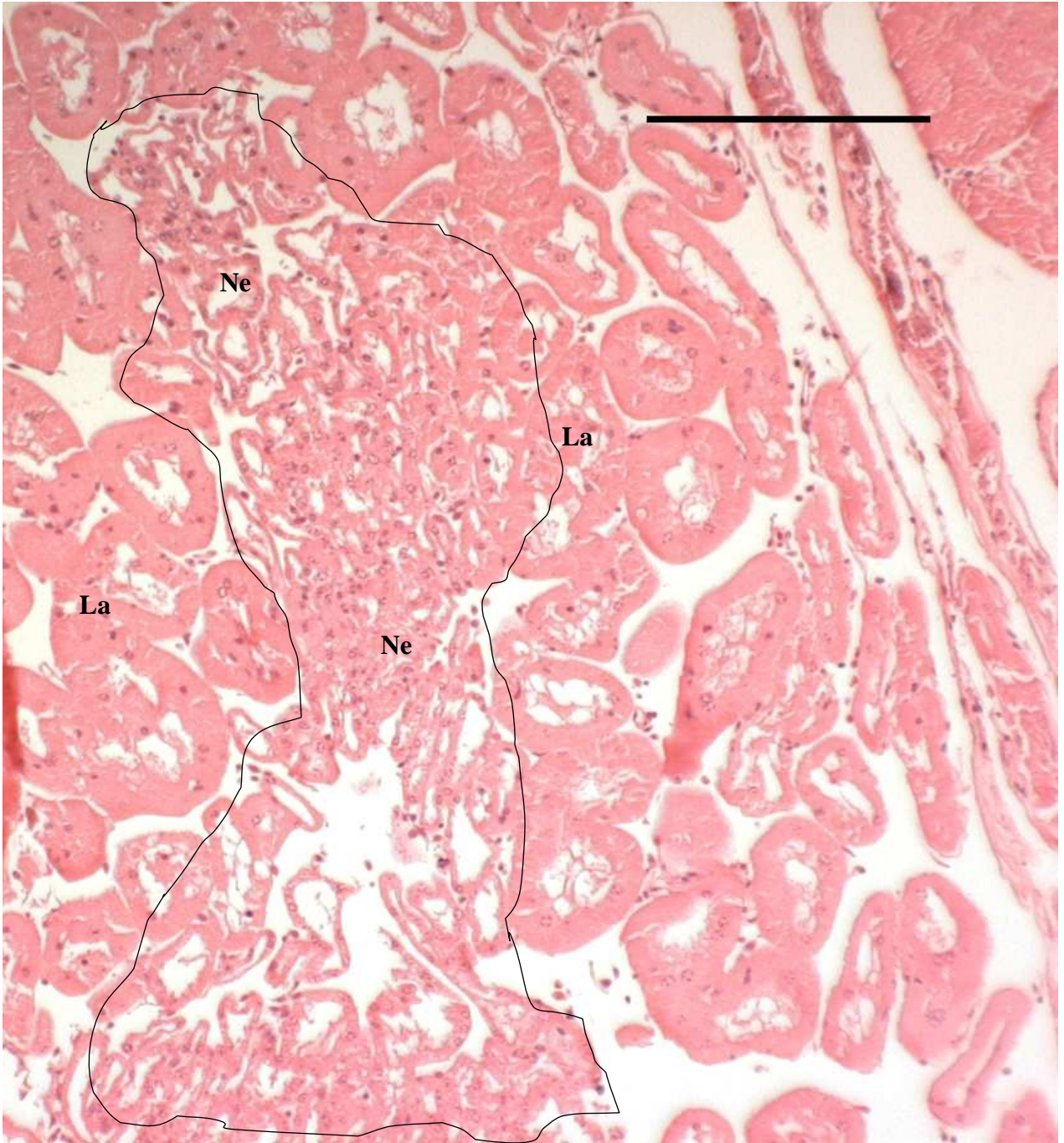


Figure 31: Light micrograph of H & E stained section showing overview location of nephridial tubule in the AG of the *M. rosenbergii*. La= labyrinth, Ne= nephridial tubule. Scale bar = 200 μ m.

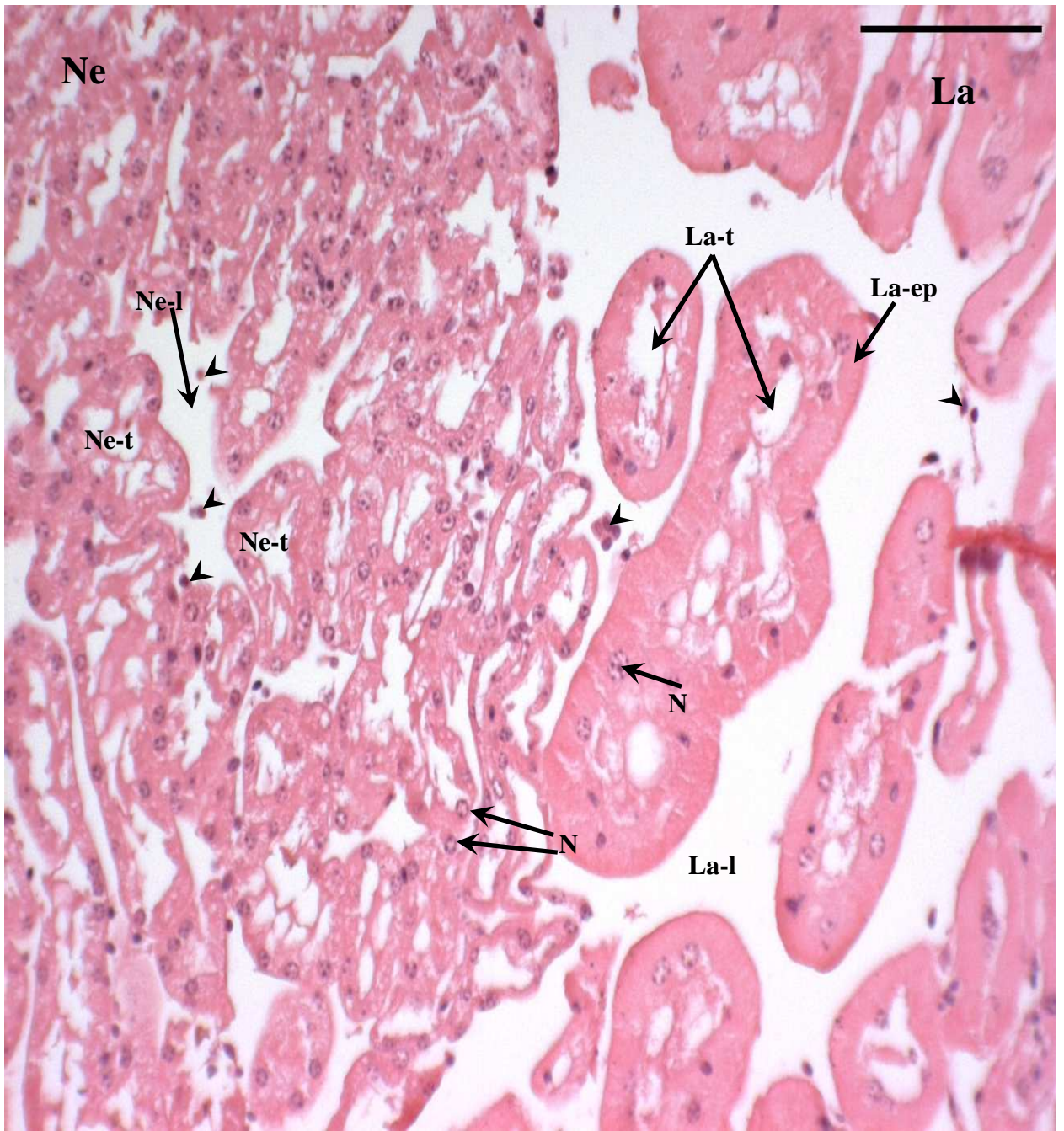


Figure 32: Light micrograph of H & E stained section showing overview location of nephridial tubule in the AG of the *M. rosenbergii*. Haemolymph vesicles (arrow heads), La= labyrinth, La-ep= labyrinth epithelium, La-l= labyrinth lumen, La-t= labyrinth tubule, N= nucleus, Ne= nephridial region, Ne-l= nephridial lumen, Ne-t= nephridial tubule. Scale bar = 200 μ m.

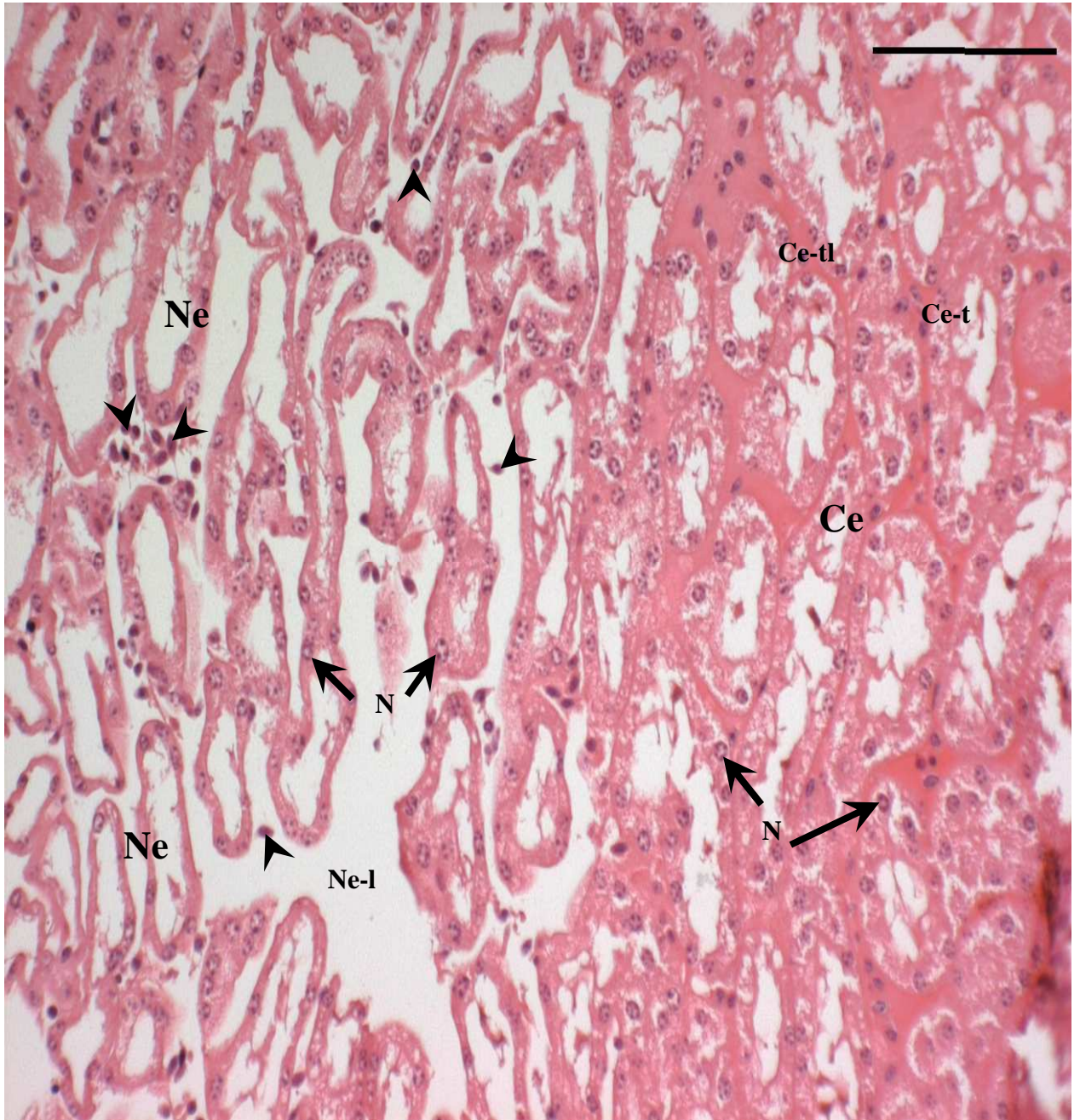


Figure 33: Light micrograph of H & E stained section showing overview location of nephridial tubule in the AG of the *M. rosenbergii*. Ce= coelomosac region, Ce-t= coelomosac tubule, Ce-l = coelomosac lumen, N= nucleus, Haemolymph vesicles (arrow heads), Ne= nephridial tubule region, Ne-l= nephridial tubule lumen, Scale bar = 250 μ m.

5.6 The ultrastructural of the AG

5.6.1 The coelomosac

As shown in Figure 34, the outer layer of the coelomosac membrane (basement membrane) contains a connective tissue with haemolymph spaces, while the inner layer of this region is lined in a large epithelial cells present in a complicated arrangement. The epithelium cells are joined together and connected to the basal membrane. The tubules in the coelomosac region is a thin epithelium layer contained a numerous number of irregular epithelium cells of podocyte. The podocyte cells have elongated and highly irregular shaped nucleus and abundant of vacuolated cytoplasm containing a dense of deposits. As shown in the Figure 33 each podocyte cell is attached to the basal membrane and project towards the lumen. The intercellular spaces usually are continuously open from the basal lamina till the luminal side. However, the most obvious features of the podocytes are the presence of large lateral channels vesicles, basal pedicels and vacuoles and intercellular dense bodies.

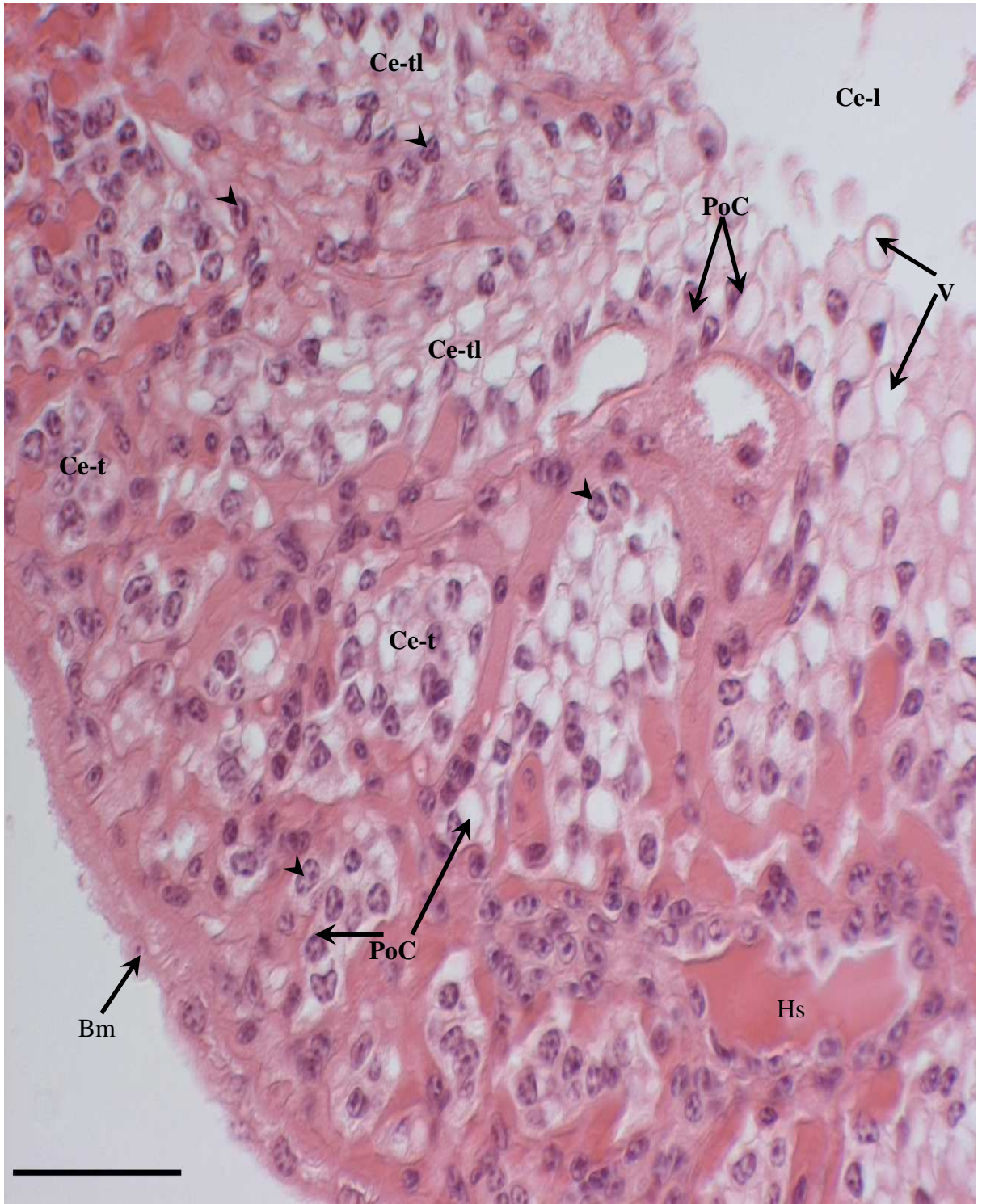


Figure 34: Light micrograph of H & E stained section showing overview part structure of the coelomosac region in the AG of the *M. rosenbergii*. Bm= basal membrane, Ce-l = coelomosac lumen, Hs = haemolymph space, Ce-t = coelomosac tubule, Ce-tl = coelomosac tubule lumen, PoC = podocyte cells, Nucleus (arrow heads), V = vacuole in podocyte cells. Scale bar = 250 μ m.

5.6.2 The labyrinth

As demonstrated above in Figures 29 and 35, the appearance of labyrinth tubules are highly coiled tubes. Labyrinth tubules formed one layer of cuboidal cells which are connective to each other and relatively thicker than the coelomosac tubules. Each cell of these tubules has dense cytoplasm, rounded or oval nuclei located in the apical side of the tubules adjacent to the lumen or sometimes central and often found in the basal side of the tubule. Also, these nucleuses have a well distinct nucleolus (dark burgundy with H&E). The apical margins of the epithelial cells of the labyrinth have well defined microvilli brush border.

Micrographs obtained by the TEM (see Figure 36) showed that cells of the labyrinth tubules are relatively large extending from the luminal side to the basal side. Generally, the TEM micrograph showed that the nuclei have a regular shape and are apically located (see Figures 35 and 37). The lumen surface is rich with long microvilli brush border and small pinocytotic vesicle could be found easily between the brush border microvilli as demonstrated in Figure 38. The mitochondria are the most prominent and abundant bodies in the labyrinth cells. They are located predominately in the central and basal sides of the cells and usually, they are either oval or elongated shaped and surrounded by the basal infoldings as shown in the Figure 38.

The cytoplasmic infoldings occupied large area of the cell and can extend for about three quarters of the distance to the luminal side. Figures 35 and 38 can demonstrate these details. The cytoplasmic membrane (at the base of the cell) creating slight slits adjacent to the basal lamina. Figure 41 gives more details about this kind of membrane.

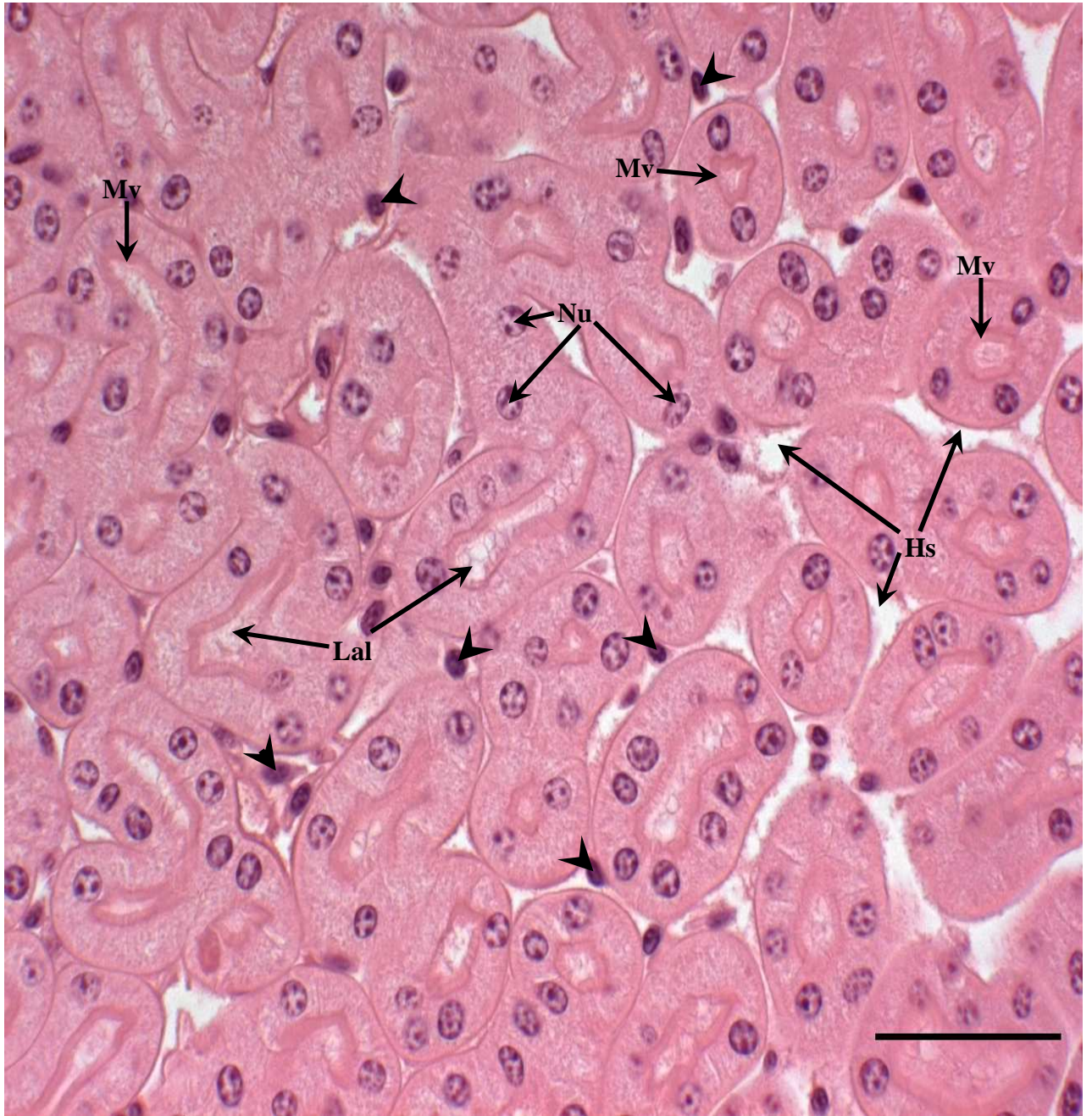


Figure 35: Light micrograph of H & E stained section showing overview structure of the labyrinth in the AG of the *M. rosenbergii*. Hs= haemolymph spaces, haemolymph vesicle (arrow heads), Lal= labyrinth lumen, Mv= microvilli border, Nu= nucleus. Scale bar = 250 μ m.

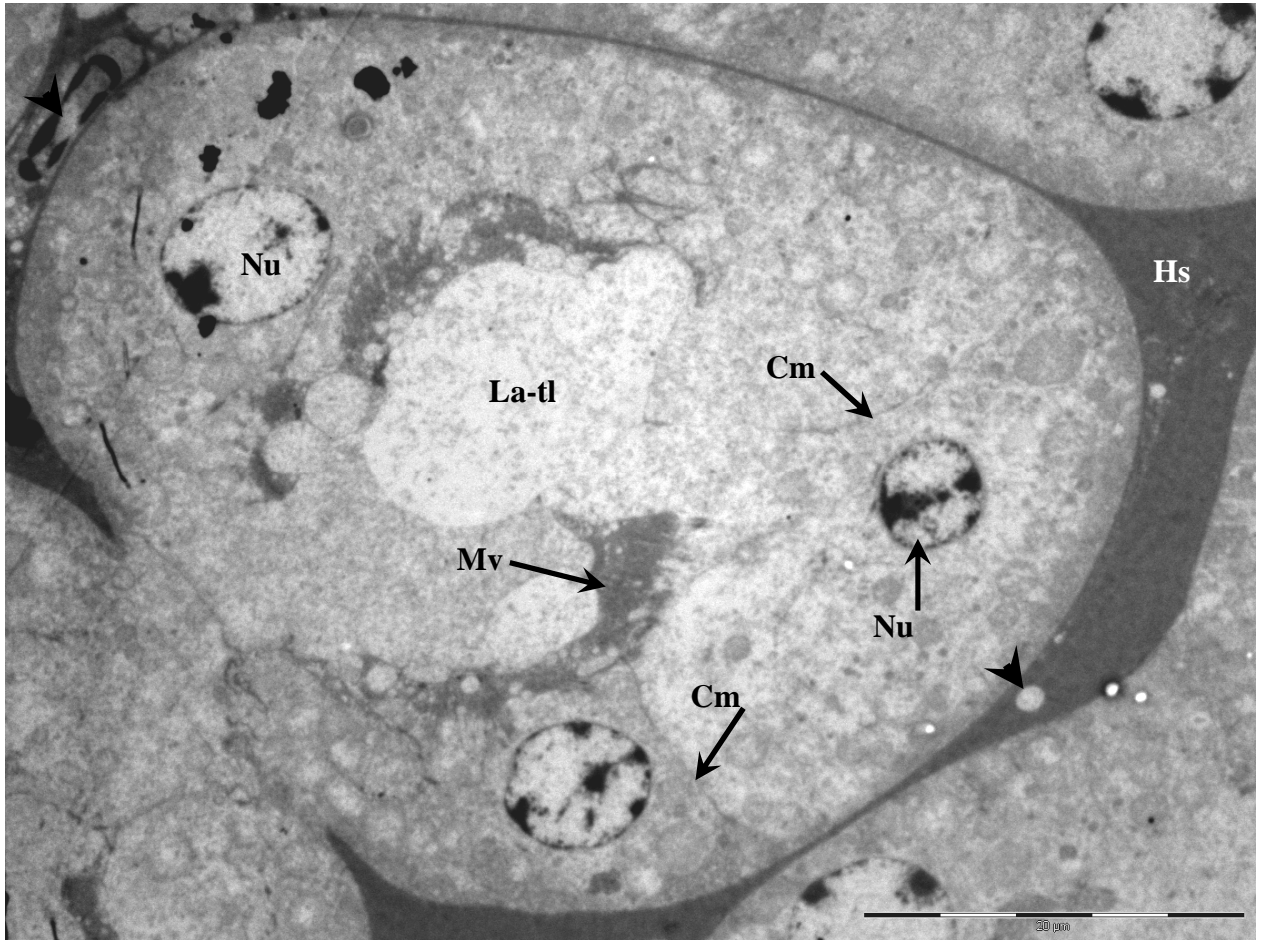


Figure 36: A TEM section showing the labyrinth-I structure in the AG of the *M. rosenbergii*. Hs= haemolymph spaces, haemolymph vesicle (arrow heads), La-tl= labyrinth tubule lumen, Mv= microvilli border, Nu= nucleus. Scale bar = 20 μm .

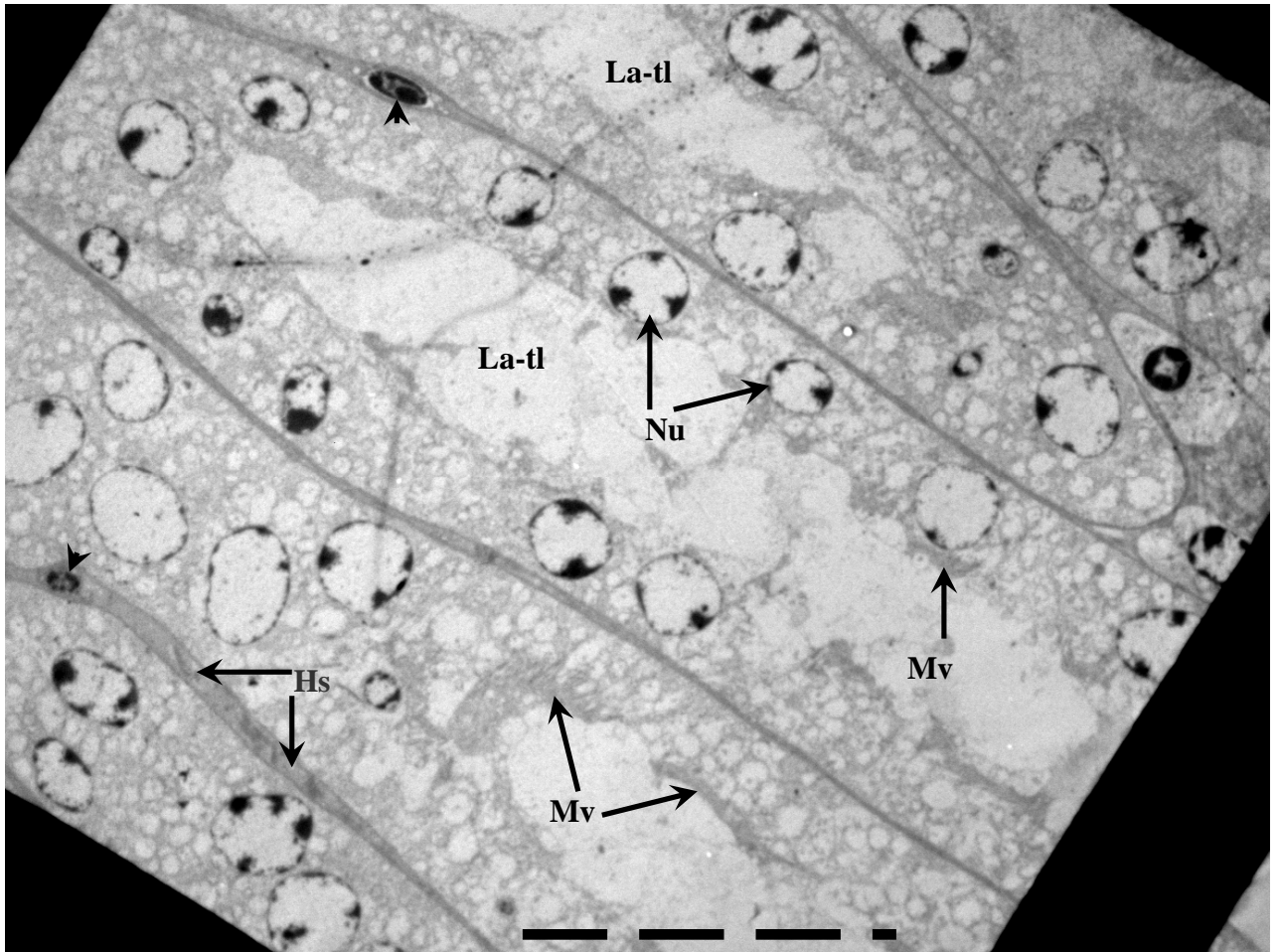


Figure 37: A TEM section showing the labyrinth-II structure in the AG of the *M. rosenbergii*. Hs= haemolymph spaces, haemolymph vesicle (arrow heads), La-tl= labyrinth tubule lumen, Mv= microvilli border, Nu= nucleus. Scale bar = 30 μ m

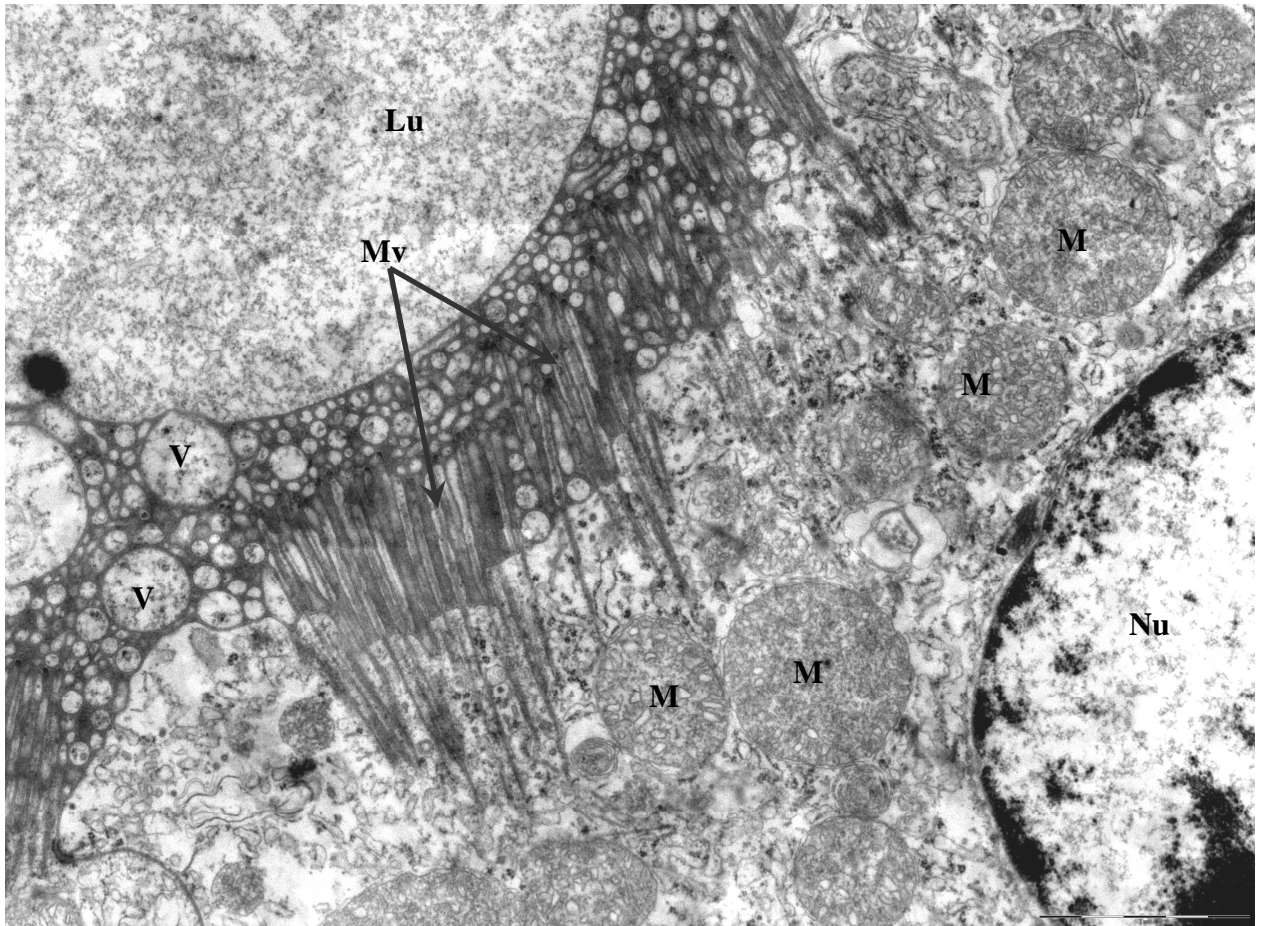


Figure 38: A TEM section showing central region of a labyrinth tubule in the AG of the *M. rosenbergii*. Lu= lumen, Mv= microvilli border, M= mitochondria, Nu= nucleus, V = vacuole. Scale bar = 2 μ m.

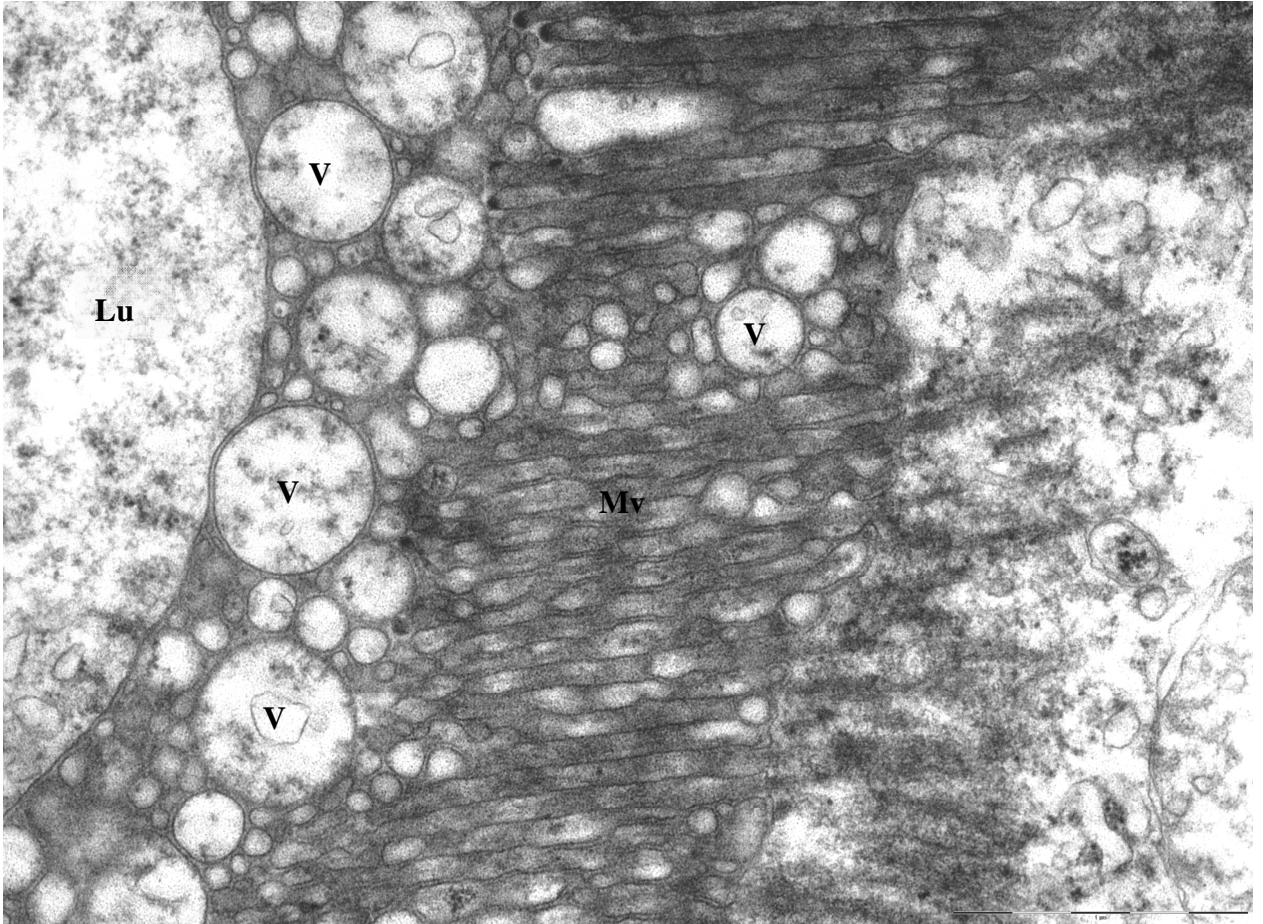


Figure 39: A TEM section showing central region of a labyrinth tubule in the AG of the *M. rosenbergii*. Lu = lumen, Mv= microvilli border, V = vacuole. Scale bar = 1 μ m.

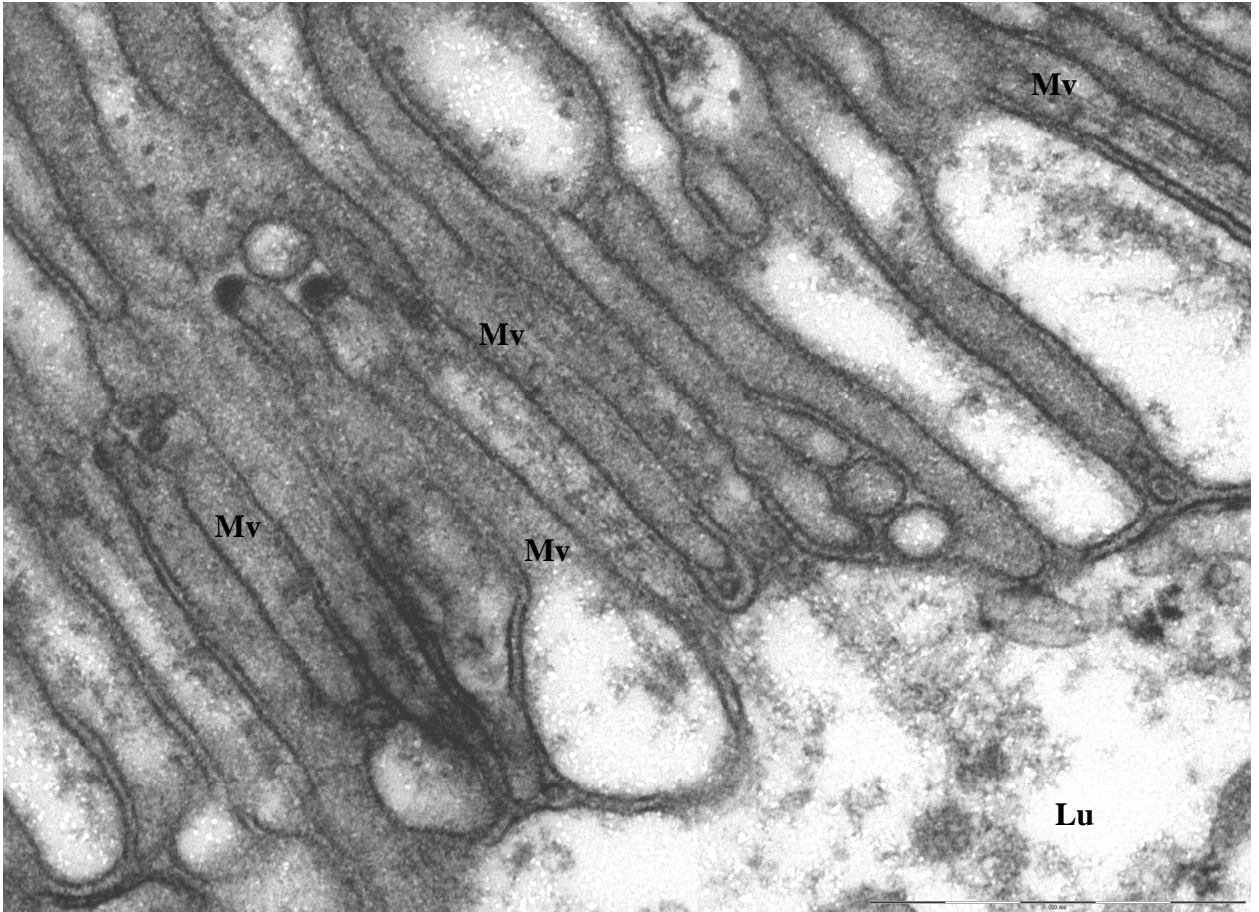


Figure 40: A TEM section showing the microvilli border in the labyrinth tubule. Mv= microvilli, Lu= lumen. Scale bar = 500 nm.

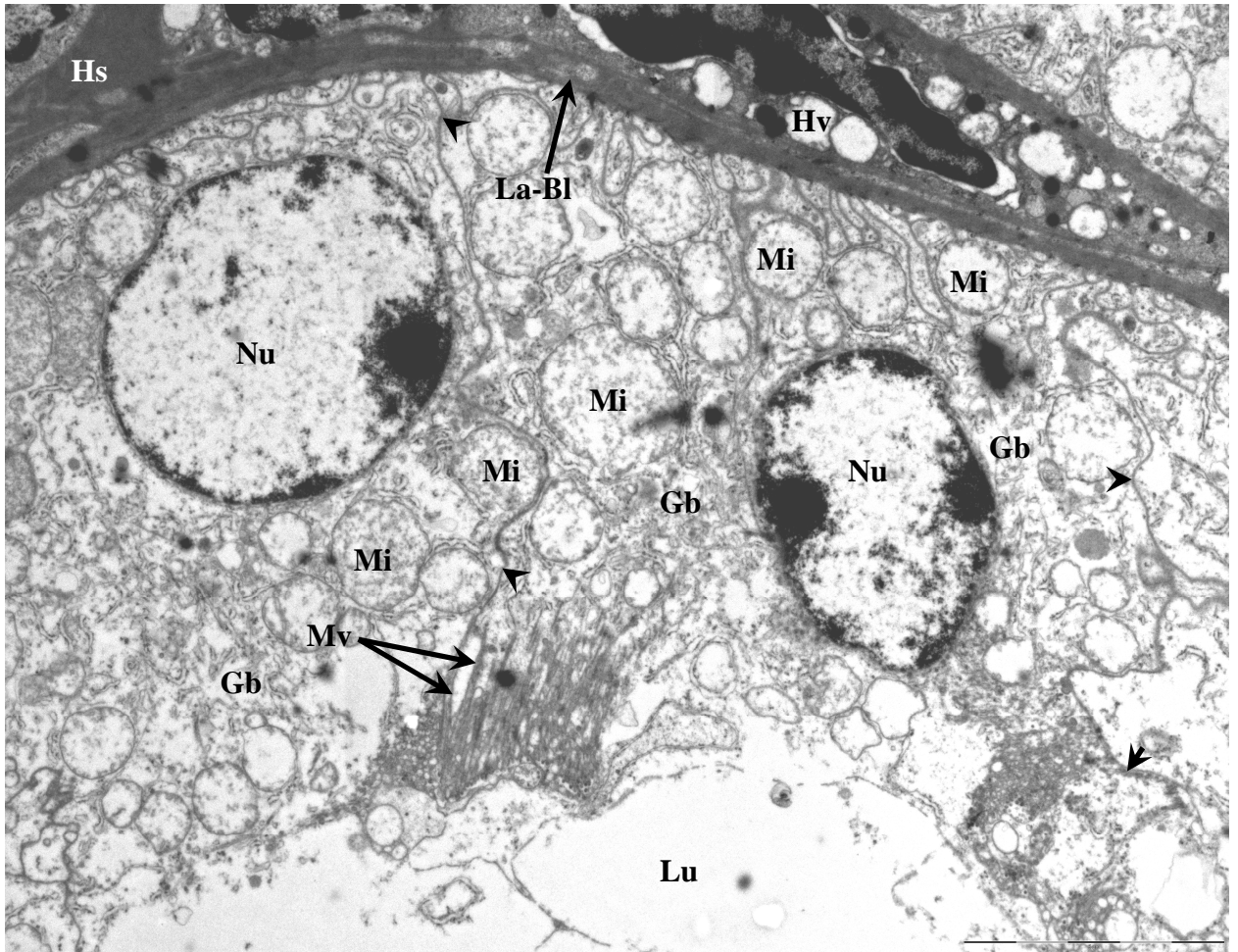


Figure 41: A TEM section showing some labyrinth tubule contents in the AG of the *M. rosenbergii*. Hv= Haemolymph vesicles, Lu= Lumen, Nu= Nucleus, Mv= Microvilli, La-BI= labyrinth basal lamina, Mi= Mitochondria, Cytoplasmic membrane junctions (arrow head), Gb=.Golgi bodies, Scale bar= 5um.

5.7 Discussion

The AG in Crustacea, including *M. rosenbergii*, is an essential excretory organ with urine being the ultimate product. The main function of the AG is to eliminate metabolic wastes, regulate haemolymph and control osmotic regulation (Vogt, 2002). Generally, the AG is located at the base of the antennae opening onto the coxa as in all decapods. This location is similar to that reported for the lobster *H. gammarus* as observed by Khodabandeh *et al.* (2005c) and crayfish (Vogt, 2002). Results obtained from the current study confirmed that urine released from *M. rosenbergii* carries a sex pheromone involved in reproductive behaviour. However, the definitive site of pheromone production remains unclear. The question therefore raises as to whether the AG is the site where sex pheromone is produced or whether another organ is responsible for producing pheromone? Thus, morphological and functional investigation of the AG in the *M. rosenbergii* is conducting.

Results from histological analysis in the present study suggested that the AG of *M. rosenbergii* has four distinct regions, the coelomosac, the nephridial tubules, the labyrinth and the bladder. This structure reflects that seen in several crustacean species such as the blue crab, *C. sapidus* (Johnson, 1980), the crayfish, *A. astacus* (Vogt, 2002), *P. clarkii* (Wheatly *et al.*, 2004), *A. leptodactylus* (Khodabandeh *et al.* (2005a), the lobster, *H. americanus* (Dove, 2005) and the shrimp, *P. monodon* (Shuang-lin and Nai-cheng, 2007). The findings of this study slightly disagree with previous works of concerning some other Crustacea such as Palaemonid prawns (Panikkar, 1941), the freshwater crayfish, *P. blandingi* (Peterson and Loizzi, 1973, 1974), *P. leniusculus* (Fuller *et al.*, 1989), the lobster, *H. americanus* (Behnke *et al.*, (1990) and the shrimp,

P. japonicus (Nakamura and Nishigaki, 1991) and *P. chinensis* (Xiaoyun *et al.*, 2003) who reported that the AG has only three regions, the coelomosac, the labyrinth and the bladder. There is more than one possible reason for these different descriptions. Among these, is the possibility of there being a nephridial tubule region present in freshwater crustacean species and absent in marine species. Also, it may be that at the time of the experiment, the observed animals might not have been in a fully developed state such that immature and small sized Crustacea might not have been possessed fully developed AGs. Another possible reason is that reports of the AG comprising three regions are erroneous, especially since some of these works were conducted early on in the past century and relied upon scientific techniques that were less developed compared to those of recent years.

5.8 The coelomosac

5.8.1 Morphology

The coelomosac region of the AG of *M. rosenbergii* was found to be posterior to the labyrinth region, this being slightly different from the location of this region in the lobster *H. gammarus* which is on the dorsal part of the labyrinth, as described by Khodabandeh *et al.* (2005c). Nevertheless, the coelomosac region in *H. gammarus* is connected directly to the bladder wall (Khodabandeh *et al.*, 2005c), as is that of *M. rosenbergii* (as shown in Figure 34).

The morphological description of the coelomosac in most described Crustacea is more or less similar. In *M. rosenbergii* described in the present study a main lumen passed through the central area of the coelomosac leading to the labyrinth. The structure of the

coelomosac in the lobster, *H. gammarus* was described by Brown (1976) and comprised a lumen, blood vessel and boundary epithelium. This lumen is also similar to that of *H. gammarus* described by Khodabandeh *et al.* (2005c) who reported that this particular lumen is the only connecting link between the coelomosac and the labyrinth for fluid transfer. However, findings in the current study showed clearly that the connection between the coelomosac and the labyrinth regions is through a duct. This differs from the observations of Johnson (1980) who reported that the opposing epithelia of the coelomosac and the labyrinth regions were joined irregularly across the haemolymph space in the blue crab, *C. sapidus*.

Podocyte cells in the coelomosac region of *M. rosenbergii* are similar to those described for other species of Crustacea such as crayfish *A. leptodactylus* (Khodabandeh *et al.*, 2005a), and their cytoplasmic features were similar to those described previously in blue crab, *C. sapidus* (Johnson, 1980) and crayfish *P. leniusculus* (Fuller *et al.*, 1989).

5.8.2 Function

The structure of the podocyte cells in the coelomosac region of *M. rosenbergii* suggests that fluid passes through the fibrous basal lamina, moving between the podocyte cell spaces (intercellular spaces) and ending up in the lumen of the coelomosac. These intercellular spaces are believed to play an important role, acting as the main route for the ultra-filtrated haemolymph, allowing transfer from the basal membrane to the apical side towards the coelomosac lumen. This is in agreement with the study conducted by Xiaoyun *et al.* (2003) who proposed that these particular cells are in charge of absorbing

the large molecules from the primary urine. However, for confirmation of these observations in *M. rosenbergii* further precise investigation is required.

Ultrastructural descriptions (Figure 28 page 190) made in this study therefore provide good evidence for the coelomosac region as the site of the filtration process in *M. rosenbergii*. This was also reported in other crustacean species including lobster, *H. americanus* (Burger, 1957), crayfish *P. leniusculus* (Kirschner and Wagner, 1965; Riegel, 1963), the freshwater crayfish, *P. blandingi* (Peterson and Loizzi, 1974), *P. clarkii* (Wheatly *et al.*, 2004) and crayfish *A. leptodactylus*. (Khodabandeh *et al.*, 2005a).

5.9 The labyrinth

5.9.1 Morphology

The labyrinth region observed in this study comprised the largest proportion of the AG in the *M. rosenbergii* as shown in the Figures 27 and 28. This was also reported in some previous studies, such as for the lobster *H. gammarus* (Khodabandeh *et al.*, 2005c). The location of the labyrinth is ventral and it lies anterior to the coelomosac region of the AG of *M. rosenbergii*. This reflects the position reported for the lobster, *H. gammarus* (Khodabandeh *et al.*, 2005c).

The histological assay in this study revealed two structurally distinct sub-regions of the labyrinth (labyrinth-I and labyrinth-II) in the AG of the *M. rosenbergii*. Labyrinth-I, relatively, represents a larger part of the labyrinth than labyrinth-II and is located next to

the coelomosac, while labyrinth-II is located in the distal part of the opposite side of the coelomosac. The lumen in labyrinth-II is wider than that in labyrinth-I of *M. rosenbergii* AG. Early studies by Peterson and Loizzi (1973, 1974) showed that the labyrinth region in the freshwater crayfish, *P. blandingi* also has two distinct parts. In more recent studies Khodabandeh *et al.* (2005c) demonstrated that the lumen of labyrinth-I of the lobster, *H. gammarus* is wider than that of labyrinth-II, which differs from *M. rosenbergii*. There is more than one possible reason for these differences. First, the histological procedure in either study could be applied differently in terms of sectional orientation. Although the histological protocol in preparing the AG of both species is similar, the structures of the AGs in both species are probably intrinsically different with the labyrinth being more dorsal in lobster than in *M. rosenbergii*. Finally, this difference may reflect structural variations between marine and freshwater species.

The labyrinth (both labyrinth-I and II) in the AG of the *M. rosenbergii* had cuboidal and columnar cells with dense microvilli at the apical side of the cells. The intercellular spaces are narrow. Such structure was described early on in the freshwater crayfish, *P. blandingi* by Peterson and Loizzi (1973, 1974). However, the labyrinth cell structure and contents may vary according to phases of secretion in the *M. rosenbergii*. Morphological features differing in the two sub-regions of the labyrinth suggest that there are likely to be some differences in their function. These observations are supported by previous studies of some other crustacean species such as the lobster, *H. gammarus* (Khodabandeh *et al.*, 2005c) and crayfish *A. leptodactylus* (Khodabandeh *et al.*, 2005a,b).

As noted in this study, the general features of the epithelial cells in the labyrinth included an apical microvillar brush border, cytoplasmic vesicles, apical cytoplasmic extrusions, cytoplasmic vacuoles and basal membrane infoldings. These features were also observed in other crustacean species such as the blue crab, *C. sapidus* Johnson (1980) and crayfish *P. leniusculus* (Fuller *et al.*, 1989).

5.9.2 Function

The majority of labyrinth tubules have a well developed microvillar border at the luminal surface which in turn suggests that this region has high absorptive capacity. The intercellular spaces between the labyrinth cells found in this study were narrow and cells are close to each other. Early, similar findings have been reported for the lobster, *H. gammarus* (Brown, 1976) and the fiddler crab, *U. mordax* (Schmidt-Nielsen *et al.*, 1986). This could suggest that these tubules are adaptive to absorptive function.

5.10 The bladder

5.10.1 Morphology

The bladder resembled a large sac surrounding the other components of the AG. At a certain point within the AG, the bladder and the labyrinth are connected by a tubule. The epithelial wall of the bladder in *M. rosenbergii* is relatively thin, comprising a single cell layer and the main lumen is substantial, occupying the majority of the bladder region. This may give flexibility for expansion during urine storage and contraction when the prawn needs to discharge urine. The epithelial layer of the bladder comprised a series of rounded cells some having cytoplasmic extrusions and large vacuoles. Microvilli were not present in the epithelial layer of the bladder. A similar

description of this epithelial layer was also given for other freshwater Crustacea such as crayfish, *A. astacus* (Vogt, 2002) and *A. leptodactylus* (Khodabandeh *et al.*, 2005a). Such similarities suggest that AGs in freshwater Crustacea share the same morphological structure in the bladder region. However, the epithelial tissue of the bladder in *M. rosenbergii* is not similar to the labyrinth tissue, in contrast to the findings of Johnson (1980) who reported that these particular tissues in the AG of blue crab, *C. sapidus* were similar in morphology whether in secretory or non-secretory conditions.

5.10.2 Function

The thin wall of the bladder probably gives this region more flexibility in expanding and contracting. It seems that storing urine before discharge through the nephropore is the major function of the bladder. Support for this suggestion was also obtained from studies by Binns (1969) and Vogt (2002). Moreover, the bladder may be used for more than storing urine, as reported by Binns (1969), who further suggested that bladder acts in reabsorbing some ions from the urine, and later Vogt (2002) reported that the bladder may have a role in dilution of urine. These hypotheses however need further investigations to be confirmed. The lack of microvilli in the bladder may indicate that filtration or reabsorption tasks are not performed in this region.

5.11 The nephridial tubule

5.11.1 Morphology

In *M. rosenbergii*, histology revealed the appearance of the nephridial region to be a dense network of coiled tubules with a lining epithelium comprising a thin layer of cells. These cells usually have a nucleus and pronounced dense basal invaginations of

cell membrane. The nephridial tubule was located in two different positions, one beside the labyrinth and the other beside the coelomosac. Support for this finding was also previously reported by Fuller *et al.* (1989) who described the morphological structure of the nephridial tubule in the AG of *P. leniusculus* and by Vogt (2002) in the crayfish *A. astacus*. However, histological analysis in the present study did not show that the nephridial tubule region in *M. rosenbergii* occupied the largest portion of the AG compared to that described by Vogt (2002) in the crayfish *A. astacus*. The nephridial tubules in *M. rosenbergii* lacked microvilli. Microvilli are also lacking in other Crustacea such as the crayfish, *P. blandingi* (Loizzi, 1973, 1974) and *A. astacus* (Vogt, 2002).

5.11.2 Function

The nephridial region comprises a dense network of coiled tubule and the thin epithelial layer of these tubules region may be employed for pumping ions actively against a concentration gradient. This function is supported by the mitochondria previously reported by Rogers and Wheatly (1997) who found that distal nephridial canal tissues are rich in mitochondria. The moult status of crustaceans may have an influence on the level of some ions in the AG such that the distal part of the nephridial tubule can function as a principal site of ion reabsorption. The study of Rogers and Wheatly (1997) may support this theory, reporting that calcium precipitation in the nephridial canal tissues of the crayfish *P. clarkii* was significantly more abundant during post-moult than during the inter-moult. It can be suggested that the nephridial tubule in *M. rosenbergii* is the primary site of ion reabsorption. Since the nephridial tubule region is absent in marine decapod species (Fingerman, 1992), the presence of this region in *M.*

rosenbergii can be considered a special adaptation to life in freshwater which enables them to produce urine isotonic to the haemolymph.

In conclusion, the AG in the *M. rosenbergii* is a major organ which acts to filter haemolymph carrying metabolic compounds and ions to produce urine to be discharged. Results of behavioural assays in the current study indicate that released urine from *M. rosenbergii* carries a distinct pheromone involved in reproductive behaviour. The quantity and the quality of urine is a significant factor in such type of behaviour. The ultimate objective of studying the AG of *M. rosenbergii* was to investigate the morphological structure of the AG and to investigate the region responsible for producing the sex pheromone. The histological analysis in this study provided a general description of main part of the AG, however, this analysis failed to find any evidence of tissue responsible for producing sex pheromone. Although, urine carries chemical cues involved in sexual response behaviours, this does not mean that the AG is the organ involved in sex pheromone production. In other words, it is most likely that sex pheromone is released from an organ (gland) other than the AGs. Alternatively, the results of the current study suggest that there could be a pair of glands one located on each side of the head adjacent to the AG whose product could be released via the nephropores, however, no direct evidence for this was found. Also, it could be suggested that a separate duct comes from each of these organs which then merges with the main urinary duct before it opens at the nephropores. Thus, pheromone released from each of these glands could mix with urine when both meet in a common duct before being discharged through the two nephropore openings. To support this theory, further histological research is required to reveal the structure and the function of these

putative pheromone glands and to study the effect of sex pheromone without the presence of urine.

Chapter 6 Conclusions

Macrobrachium rosenbergii is a key species for commercial aquaculture and is highly amenable to laboratory research studies. By restricting both visual and tactile communication cues, the bioassay system designed as part of this study functioned ideally for testing chemical communication between individual *M. rosenbergii* and achieved reliable results under laboratory conditions. Findings in the current study confirmed that chemical communication play an important role within *M. rosenbergii* population and playing is a key component in maintaining social hierarchy and governing reproductive behaviour. The results of the current study also clearly indicate the relationship between moult stages and sexual attraction in *M. rosenbergii*. Newly-moulted female prawns were significantly more attractive than both pre- and inter-moult females to dominant BC males. In contrast, the inter-moult BC males were significantly the more attractive to newly-moult female prawns than pre- and newly-moulted BC males. Newly-moulted females paid less attention to newly-moulted BC males, possibly because they were trying to avoid potential costs of mate guarding with soft shell BC males. Thus, it can be suggested that moult stages for males and females plays an important role in sexual attraction with both males and females capable of discriminating the moult status of the opponents from pheromone signature. It was observed that ovarian maturation and moulting stage of female were closely related. Also, characteristics of sex pheromone released from females clearly changed with ovarian maturation. Thus, moult stage, especially for the female prawns, provides an indirect indicator of sexual readiness for mating. This suggests that BC male sexual attraction behaviour may be based upon a female's ovarian maturation rather than female's moulting. Since female with fully ripe ovaries are more attractive to the BC male just prior to or during moulting, this suggests the possibility of a further

pheromone source other than that carried in urine or the presence of an additional urine-pheromone which is directly related to the ovarian maturation status.

Male morphotype (SM, OC and BC) also plays a significant role in sexual attraction. Significantly, BC males are the most preferred to newly-moulted females whilst OC males are least attractive. This suggests that both SM and BC males are sexual active while OC are far less sexually active. Female prawns can also distinguish male morphotypes from their pheromone signature. Thus, released pheromone is an essential chemical cue involved in sexual attraction between male and female prawns. The variation in female behavioural responses with respect to the different male morphotypes reflects the high activity of pheromone cues released from the BC males over OC and SM males.

Urine in the freshwater prawn is believed to be one of major pheromone carriers which facilitating chemical communication among prawn individuals. Sexual responses of newly-moulted female prawns were directly dependent upon urine concentration such that the more urine there was present, the faster the observed response. Urine concentration therefore plays a significant role in sexual attraction. Results from this study suggests that $3.0 \mu\text{l l}^{-1}$ of fresh urine is a sufficient concentration to elicit a significant sexual attraction under the laboratory conditions. From these results it can therefore be suggested that pheromone released from both male and female freshwater prawns plays several different roles in male-female interactions. It can provide multiple pieces of information on female's receptivity, on the sender's gender and on a male's social hierarchy status. Because pheromone plays a significant role in controlling the

social structure and the reproductive attraction behaviour, it becomes a very promising chemical as a tool for population culture control.

Exposing fresh urine to different temperature treatments (cooling at 4°C, freezing at -70°C and heating at 70°C) allows investigation of the characteristics of contained pheromones. Compare to fresh urine, slowest sexual responses were observed in newly-moulted female toward heated urine whereas they responded relatively faster toward cooled urine 4°C. It can be suggested that part degradation of some pheromone content in stored cooled urine and more degradation in both frozen and heated urine occur respectively. Thus, the conclusion may be drawn that pheromone in prawn urine are temperature sensitive being friable at the high and low temperatures examined.

The antennal glands (AGs) of *M. rosenbergii* are the principle excretory organs acting of discharge metabolic wastes in urine and functioning in Osmoregulation. Histological analysis revealed that the AG of the *M. rosenbergii* had four distinct regions, the coelomosac, the nephridial tubules, the labyrinth and the bladder. These four regions structure have been found in several freshwater crustacean species. The location of the coelomosac was found to be posterior to the labyrinth and connected directly to the labyrinth through a duct. The coelomosac had a simple epithelium with luminal microvillar brush border. Podocytes in *M. rosenbergii* were similar to those of other freshwater Crustacea with the intercellular spaces between podocytes cells playing an important role in ultra-filtration of haemolymph allowing fluid to transfer from the basal membrane to the apical side towards the coelomosac lumen. It is suggested that podocytes cells are responsible for reabsorbing large molecules from primary urine.

The labyrinth region of *M. rosenbergii* observed in this study comprised the largest proportion of the AG and its location was ventral and anterior to the coelomosac region. It had two structurally distinct sub-regions, labyrinth-I and labyrinth-II. Labyrinth-I was located next to the coelomosac, while labyrinth-II was located in the distal part of the opposite side of the coelomosac. In term of size, labyrinth-I is larger than labyrinth-II. However, the lumen in labyrinth-II is wider than that of labyrinth-I. These morphological differences may indicate differences in their functions. Both labyrinth-I and II had cuboidal and columnar cells with dense microvilli at the apical luminal side of the cells and the intercellular spaces are narrow. Differences in cell structure and contents may depend on secretion status. Apical microvilli, cytoplasmic vesicles, apical cytoplasmic extrusions, cytoplasmic vacuoles and basal membrane infoldings were the general features of the epithelial cells of the labyrinth. The well developed microvillar border at the luminal surface suggests that this region had a high absorptive capacity. The intercellular spaces between labyrinth cells were narrow and cells were close to each other. This could suggest that labyrinth tubules are adaptive to an absorptive function.

The nephridial region comprised a dense network of twisted tubules. The epithelium of these tubules comprised a thin layer of cells with each cell possessing a nucleus and pronounced dense basal invaginations of cell membrane. The nephridial region is located in two different positions, one beside the labyrinth and the other beside the coelomosac. The abundance of mitochondria in this region suggests that may function to pump ions against a concentration gradient and it is suggested that the distal part of the nephridial tubule can act as a key site for ion reabsorption. The moult status of *M. rosenbergii* may influence the dynamic circulation of some ions in the body with the

AGs performing in controlling the concentration balance of these ions between internal and external body. The presence of nephridial tubules is believed to be an adaptation to life in freshwater for a number Crustacea such as *M. rosenbergii* which enables them to produce urine which is isotonic to the haemolymph.

The final part of the antennal gland, the bladder, was a large sac encircling the other parts of the AG and connected with the labyrinth via a tubule. It had a thin epithelial wall consisting of a single cell layer comprising a sequence of rounded cells, some having cytoplasmic extrusions and large vacuoles. Also, there was a substantial main lumen which occupied a large mass of the bladder region which in turn gave it flexibility for expansion during urine storage. Microvilli were not present in the epithelial layer of the bladder. Such structure suggests that storing urine before discharge through the nephropore is the major function of the bladder. The bladder may also act in reabsorbing some ions from the urine and may also have a role in dilution of urine.

Given that no structures were observed that had the characteristics of glandular secretory tissues, it seems that the AGs themselves are not the site of secretion for the reported sex pheromone and it is suggested other glands located to the AG are responsible for producing the sex pheromone.

Further research to study the effect of moult status on AG morphology and the precise function of the AGs is clearly warranted. This study also prompts further analysis of the

relationship between the sexual attraction responses of *M. rosenbergii* and other biological / environmental factors and research examining the identity and site production of the pheromones whose activity has been described in this thesis.

References

- Acquistapace, P., Aquiloni, L., Hazlett, B. A. and Gherardi, F. 2002. Multimodal Communication in Crayfish: Sex Recognition During Mate Search by Male *Austropotamobius pallipes*. *Can. J. Zool.*, 80 (11): 2041 – 2045.
- Akiyama, D. Brock, J. and Harley, S. 1982. Idiopathic muscle necrosis in the cultured freshwater prawn (*Macrobrachium rosenbergii*). *Vet. Med. Small Anim. Clin.*, July, p. 1119.
- Alagarswamy, K. 1995. India-Country Report. In: FAO/NACA Report on a Regional Study and Workshop on the Environmental Assessment and Management of Aquaculture Development. NACA Environmental series No. 1. pp. 141 – 186. Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand.
- Alcock, J., Jones, E. and Buchmann, L. 1977. Male mating strategies in the bee *Centris pallida* Fox (Anthrophridae: Hymenoptera). *Am. Nat.*, 111: 145 – 155.
- Ali, P. K. M. M. and Rao, G. P. S. 1989. Growth improvement in carp, *Cyprinus carpio* (Linnaeus), sterilized with 17 α -methyltestosterone. *Aquaculture*, 76: 157 – 167.
- Ameyaw-Akumfi, C. and Hazlett, B. A. 1975. Sex recognition in crayfish *Procambarus clarkii*. *Science*, NY. 190: 1225 – 1226.
- Anderson, E. and Beams, H. W. 1956. Light and Electron Microscope Studies on the Cells of the Labyrinth in the “Green Gland” of *Cambarus* Sp. *Proc. Iowa Acad. Sci.*, 63; 681 – 685.
- Arkarajamon, A. 1991. Histology of Tiger Shrimp (*Penaeus monodon* Fabricius). Master Thesis, Kasetsart University. 168 p.
- Arndt, G. D., Gautz, L. D. and Wang, Jaw-Kai. 1984. Mechanical size grading of postharvest prawns. *J. World Maricul. Soc.*, 15: 61 – 72.
- Arrignon, J. C. V., Huner, J. V., Laurent, P. J., Griessinger, J. M., Lacroix, D., Gondouin, P. and Autrand, M. 1994. Warm-Water Crustaceans, In: *The tropical Agriculturalist*, Coste, R. Ed. MacMillan Press Ltd., pp. 160. London, UK.
- Atema, J. 1985. Chemoreception in the sea: adaptations of chemoreceptors and behaviour to aquatic stimulus conditions. *Symp. Sco. Exp. Biol.* 39: 387 – 423.
- Atema, J. 1986. Review of sexual selection and chemical communication in the lobster, *H. americanus*. *Can. J. Fish. Aquat. Sci.*, 43: 2283 – 2290.
- Atema, J. 1995. Chemical signals in the marine environment: dispersal, detection, and temporal signal analysis. *Proc. Natl. Acad. Sci. USA.* 92: 62 – 66.
- Atema, J. and Cobb, J. S. 1980. Social behaviour, p. 409 - 450. In: Cobb, J. S. and Phillips, B. F. (Eds.). *The biology and management of lobsters*. Vol. 1 Academic Press, New York, USA.

- Atema, J. and Cowan, D. F. 1986. Sex-identifying urine and molt signals in lobster (*Homarus americanus*). *Journal of Chemical Ecology*, 12 (11): 2065 – 2080.
- Atema, J. and Engstrom, D. G. 1971. Sex pheromone in the lobster, *Homarus americanus*. *Nature*, 232: 261 – 263.
- Atema, J., Jacobson, S., Karnofsky, E. B., Oleszko-Szuts, S., and Stein, L. 1979. Pair formation in the lobster, *H. americanus*: Behavioral development, pheromones and mating. *Mar. Behav. Physiol.*, 6: 277 – 296.
- Atema, J and Voigt, R. 1995. Behaviour and Sensory Biology. In: *Biology of the Lobster H. americanus*. (Jan Robert Factor, Ed.). Pages 313 – 348. Academic Press, Inc. San Diego, California, USA.
- Austad, S. N. 1984. A Classification of Alternative Reproductive Behaviours and Methods for Field – testing ESS Models. *Amer. Zool.*, 24: 309 – 319.
- Avault, J. W. 1987. Species profile – freshwater prawns and marine shrimp. *Aquaculture magazine*, 13 (3): 53 – 56.
- Baghel, D. S., Lakra, W. S. and Rao, G. P. S. 2004. Altered sex ratio in giant fresh water prawn, *Macrobrachium rosenbergii* (de Man) using hormone bioencapsulated live *Artemia* feed. *Aquaculture Research*, 35: 943 – 947.
- Bamber, S. D., and Naylor, E. 1996a. Chemical communication and behavioural interaction between sexually mature male and female shore crabs (*Carcinus maenas*). *J. Mar. Biol. Ass., UK*. 76: 691 – 699.
- Bamber, S. D., and Naylor, E. 1996b. Mating behaviour of male *Carcinus maenas* in relation to a putative sex pheromone: behavioural changes in response to antennule restriction. *Marine Biology*, 125 (3): 483 – 488.
- Bamber, S. D., and Naylor, E. 1997. Site of release of putative sex pheromone and sexual behaviour in female *Carcinus maenas* (Crustacea: Decapoda). *Estuarine, Coastal and Shelf Science*, 44: 195 – 202.
- Barki, A., Karplus, I., and Goren, M. 1991. The agonistic behaviour of the three male morphotypes of the freshwater prawn *Macrobrachium rosenbergii* (Crustacea, Palaemonidae). *Behaviour*, 116: 252 – 277.
- Barki, A., Karplus, I., and Goren, M. 1992. Effects of size and morphotype on dominance hierarchies and resource competition in the freshwater prawn *Macrobrachium rosenbergii*. *Animal Behaviour*, 44 (3): 547 – 555.
- Barth, R. H., JR. 1961. Hormonal control of sex attractant production in the Cuban cockroach. *Science*, 133: 1598 – 1599.
- Bauer, R. T. 1976. Mating behaviour and spermatophore transfer in the shrimp *Hepatcarpus pictus* (Stimpson) (Decapoda: Caridea: Hippolytidae). *J. Nat. Hist.*, 10: 415 – 440.

- Bauer, R. T. 1979. Sex attraction and recognition in the Caridean shrimp *Hepatcarpus paludicola* Holmes (Decapoda: Hippolytidae). *Mar. Behav. Physiol.*, 6: 157 – 174.
- Bauer, R. T. and Abdalla, J.H. 2001. Male mating tactics in the shrimp *Palaemonetes pugio* (Decapoda, Caridea): precopulatory mate guarding vs. pure searching. *Ethology*, 107: 185 – 199.
- Behrends, L. L., Kingsely, J. B. and Price, A. H. 1986. Polyculture of freshwater prawns, tilapia, channel catfish and Chinese carps. *J. World Maricult. Soc.*, 16: 437 – 450.
- Benhke, R. D. Wong, R. K. Huse, S. M. Reshkin, S. J. and Ahearn, G. A. 1990. Proline transport by brush-border membrane vesicles of lobster antennal glands. *American Physiological Society, Renal Physiology*, 258 (2): 311 – 320.
- Bergman, D. A., Kozloewski, C. P., McIntyre, J. C., Huber, R., Daws, A. G. and Moore, P. A. 2003. Temporal dynamics and communication of winner-effects in the crayfish, *Orconectes rusticus*. *Behaviour*, 140: 805 – 825.
- Bergman, D. A., Martin, A. L. and Moore, P. A. 2005: Control of information flow through the influence of mechanical and chemical signals during agonistic encounters by the crayfish, *Orconectes rusticus*. *Anim. Behav.*, 70: 485 – 496.
- Bhatt, J. P., Kandwal, J. S. and Nautiyal, R. 2002. Water temperature and pH influence olfactory sensitivity to pre-ovulatory and post-ovulatory ovarian pheromones in male *Barilius bendelisis*. *J. Biosci.*, 27 (3): 273 – 281.
- Binns, B. and Peterson, A. J. 1969. Nitrogen excretion by the spiny lobster *Jasus edwardsi* (Hutton): The role of the antennal gland. *Biological Bulletin*, 136: 147 – 153.
- Bjerselius, R. Olsén, K. H. and Zheng, W. 1995. Behavioural and Endocrinological Responses of Mature Male Goldfish to the Sex Pheromone 17a,20b-Dihydroxy-4-Pregnen-3-One in the Water. *The Journal of Experimental Biology*, 747 (198): 747 – 754.
- Borowsky, B. 1984. Effects of receptive females' secretion on some male reproductive behaviours in the amphipod crustacean *Microdeutopus gryllotalpa*. *Marine Biology*, 84 (2): 183 – 187.
- Borowsky, B. 1985a. Differences in reproductive behaviour between two male morphs of the amphipod crustacean, *Jassa falcata Montagu*. *Physiological Zoology*, 58: 497 – 502.
- Borowsky, B. 1985b. Responses of the amphipod crustacean *Gammarus palustris* to water-borne secretions of conspecifics and congenics. *Journal of Chemical Ecology*, 11 (11): 1545 – 1552.
- Borowsky, B. 1991. Patterns of reproduction of some amphipod crustaceans and insights to the nature of their stimuli. In, *Crustacean Sexual Biology*, edited by

- E.T. Bauer and J. W. Martin, Columbia University Press, NY, Ch. 3, pp. 34 – 49.
- Borowsky, B. C., Augelli, E. and Wilson, S. R. 1987. Towards chemical characterization of water-borne pheromone of amphipod crustacean *Microdeutopus gryllotalpa*. *Journal of Chemical Ecology*, 13 (7): 1673 – 1680.
- Bouchard, S. Sainte-Marie, B. and McNeil, J. N. 1996. Indirect evidence indicates female semiochemicals release male precopulatory behaviour in the snow crab, *Chionoecetes opilio* (Brachyura: Majidae). *Chemoecology*, 7 (1): 39 – 44.
- Bovbjerg, R. V. 1970. Ecological isolation and competitive exclusion in two crayfish (*Orconectes virilis* and *Orconectes immunis*). *Ecology*, 51: 225 – 236.
- Boyd, C. and Zimmermann, S. 2000. Grow-out systems – water quality and soil management. In M.B. New and W.C. Valenti, eds. *Freshwater prawn culture: the farming of *Macrobrachium rosenbergii**, pp. 221 – 238. Oxford, England, Blackwell Science.
- Bradbury, J. W. and Vehrencamp, S. L. 1998. *Principles of Animal Communication*. Sunderland, MA, USA: Sinauer Association, Inc. Publishers. 994pp.
- Breithaupt, T. 2001. Fan Organs of Crayfish Enhance Chemical Information Flow. *Biol. Bull.*, 200: 150 – 154.
- Breithaupt, T. and Atema, J. 1993. Evidence for the Use of Urine Signals in Agonistic Interactions of the American Lobster. *Biological Bulletin*, 185: 318.
- Breithaupt, T. and Atema, J. 2000. The timing of chemical signalling with urine in dominance fights of male lobsters (*Homarus americanus*). *Behavioral Ecology and Sociology*, 49 (1): 67 – 78.
- Breithaupt, T. and Eger, P. 2002. Urine makes the difference: chemical communication in fighting crayfish made visible. *J. Exp. Biol.*, 205: 1221 – 1231.
- Breithaupt, T., Lindstrom, D. P. and Atema, J. 1999. Urine release in freely moving catheterised lobsters (*Homarus americanus*) with reference to feeding and social activities. *J. Exp. Biol.*, 202: 837 – 844.
- Briggs, R. T. and Moss, B. I. 1997. Ultrastructure of the Coxal Gland of the Horseshoe Crab *Limulus polyphemus*: Evidence for Ultrafiltration and Osmoregulation. *Journal of Morphology*, 234: 233 – 252.
- Brody, T., Cohen, D., Barnes, A. and Spector, A. 1980. Yield characteristics of *Macrobrachium rosenbergii* in temperate zone aquaculture. *Aquaculture*, 21: 375 – 385.
- Brown, J. H. 1976. A study of the Excretion of Inorganic Mercury, and the Long Term Effects of Exposure to Low Levels of the Metal in the Lobster, *Homarus gammarus* (L) White 1847. A PhD thesis, The University of St Andrews.

- Brown, J. H. 1991. Freshwater Prawns. In: Nash, C.E., (Ed.), World Animal Science, Production-System Approach, Production of Aquatic Animals, Crustaceans, Molluscs, Amphibians and Reptiles - C4, Amsterdam, The Netherlands, pp. 31 – 42.
- Brown, J. H., Wickins, J. F. and MacLean, M. H. 1991. The effect of water hardness on growth and carapace mineralization of juvenile freshwater prawns, *Macrobrachium rosenbergii* de Man. *Aquaculture*, 95: 329 – 345.
- Bruski, C. A. and Dunham, D. W. 1987. The Importance of Vision in Agonistic Communication of the Crayfish *Orconectes rusticus*. I An Analysis of Bout Dynamics. *Behaviour*, 103 (1–3): 83 – 107.
- Bruski, C. A. and Dunham, D. W. 1990. Antennal waving in the crayfish *Orconectes rusticus* (Girard, 1852) (Decapoda, Astacidae). *Crustaceana*, 58: 83 – 87.
- Buck, D. H., Malecha, S. R. and Baur, R. J. 1981. Polyculture of the freshwater prawn *Macrobrachium rosenbergii* with two combinations of carps in manured ponds. *J. World Maricult. Soc.*, 12 (2): 203 – 213.
- Burger, J. W. 1957. The general form of excretion in the lobster, *Homarus*. *The Biological Bulletin*, 113 (2): 207 – 223.
- Burrs, R. L. and Lodge, D. M. 2002. Cued In: Advances and Opportunities in Freshwater Chemical Ecology. *Journal of Chemical Ecology*, 28 (10): 1901 – 1917.
- Bushmann, P. J. 1999. Concurrent signals and behavioral plasticity in blue crab (*Callinectes sapidus* Rathbun) courtship. *Biological Bulletin*, 197: 63 – 71.
- Bushmann, P. J. and Atema, J. 1993. A Novel Tegumental Gland in the Nephropore of the Lobster. *Homarus americanus*: A site for the Production of Chemical Signals. *Biological Bulletin*, 185: 319 – 320.
- Bushmann, P. and Atema, J. 1994. Aggression-reducing courtship signals in the lobster, *Homarus americanus*. *Biol. Bull.*, 187: 275 – 276.
- Bushmann, P. J and Atema, J. 1996a. Chemical communication signals as determinants of mating success in the lobster, *Homarus americanus*. Twenty-Fourth Annual Benthic Ecology Meeting, Held in Columbia, South Carolina, USA, March 7 – 10, 1996, P. 24.
- Bushmann, P. J. and Atema, J. 1996b. Nephropore rosette glands of the lobster *Homarus americanus*: possible sources of urine pheromones. *Journal of Crustacean Biology*, 16 (2): 221 – 231.
- Bushmann, P. J. and Atema, J. 1997. Shelter sharing and chemical courtship signals in the lobster, *Homarus americanus*. *Can. J. Fish. Aquat. Sci.*, 54 (3): 647 – 654.
- Bushmann, P. J. and Atema, J. 2000. Chemically mediated mate location and evaluation in the lobster, *Homarus americanus*. *Journal of Chemical Ecology*, 26: 647 – 654.

- Caldwell, R. L. 1979. Cavity occupation and defense behaviour in the Stomatopod *Gondactylus festai*; evidence for chemically mediated individual recognition. *Animal Behaviour*, 27: 194 – 201.
- Chang, C. E., Lee, M. F. and Chen, G. R., 1994. Estradiol-17 β associated with the sex reversal in protandrous black progy, *Acanthopagrus schlegeli*. *J. Exp. Zool.*, 268: 53 – 59.
- Chang, C. F. and Shih, T. W. 1995. Reproductive cycle of ovarian development and vitellogenin profiles in the freshwater prawns, *Macrobrachium rosenbergii*. *Invertebrate Reproduction and Development*, 27 (1): 11 – 20.
- Charniaux-Cotton, H. 1965. Hormonal control of sex differentiation in invertebrates. In: Hann, R.D. and Ursprung, H. (Eds.) *Organogenesis*. Holt, New York, pp. 701 – 740.
- Charniaux-Cotton, H., Payen, G. 1985. Sexual differentiation. In: Bliss, D.E., Mantel, L.H. (Eds.), *Integument, Pigments, and Hormonal Processes*. Academic Press, New York, pp. 290 – 330.
- Chavez Justo, C. Aida, K. and Hanyu, I. 1991. Effects of photoperiod and temperature on molting, reproduction and growth of the freshwater prawn *Macrobrachium rosenbergii*, *Nippon Suisan Gakkaishi*, 57: 209 – 217.
- Chew, K. 1990. Shao-Wen Ling 1907-1990. *World Aquaculture*, 21 (4): 31.
- Chow, S., Ogasawara, Y., and Taki, Y. 1982. Male reproduction system and fertilisation of the palaemoid shrimp *Macrobrachium rosenbergii*. *Bull. Jpn. Soc. Sci. Fish.*, 48 (2): 177 – 183.
- Chow-Fraser, P. and Maly, E. J. 1988. Aspects of mating, reproduction, and co-occurrence in three freshwater calanoid copepods. *Freshwater Biol.*, 19: 95 – 108.
- Christofferson, J. P., 1978. Evidence for the controlled release of a crustacean sex pheromone. *Journal of chemical Ecology*, 4: 633 – 639.
- Christy, J. H., and Salmon, M. 1991. Comparative studies of reproductive behaviour in mantis shrimps and fiddler crabs. *Am. Zool.*, 31: 329 – 337.
- Christy, J. H., Backwell, P. R. Y. and Schober, U. 2003. Interspecific attractiveness of structures built by courting male fiddler crabs: experimental evidence of a sensory trap. *Behav. Ecol. Sociobiol.*, 53: 84 – 91.
- Clark, R. A. 1997. Dimorphic male display alternative reproductive strategies in the marine amphipod *Jassa marmorata* Holmes (Corophioidea: Ischyroceridae). *Ethology*, 103: 531 – 553.
- Cobb, J.S., Tamm, G.R. and Wang, D. 1982. Behavioral mechanisms influencing molt frequency in the American Lobster, *H. americanus* Milne Edwards. *Journal of Experimental Marine Biology and Ecology*, 62: 185 – 200.

- Cohen, D. and Ra'anan, Z. 1983a. The production of the freshwater prawn *Macrobrachium rosenbergii* in Israel. III. Density effect of all-male Tilapia hybrid on prawn yield characters in polyculture. *Aquaculture*, 35: 57 – 71.
- Cohen, D., Ra'anan, Z. and Brody, T. 1981 Population profile development and morphotypic differentiation in the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man). *Journal of the World Mariculture Society*, 12 (2): 231 – 243.
- Cohen, D., Ra'anan, Z., Rappaport, U. and Arieli, Y. 1983b. The production of the freshwater prawn *Macrobrachium rosenbergii* (de Man) in Israel. Improved conditions for intensive monoculture. *Bamidgeh*, 35 (2): 31 – 37.
- Cohen, D., Sagi, A., Ra'anan, Z. and Zohar, G. 1988. The production of *Macrobrachium rosenbergii* in monosex populations, III. yield characteristics under intensive monoculture conditions in earthen ponds. *Bamidgeh*, 40(2): 57 – 63.
- Correa, C., and M. Thiel. 2003a. Population structure and operational sex ratio in the rock shrimp *Rhynchocinetes typus* (Decapoda: Caridea). *J. Crustac. Biol.*, 23: 849 – 861.
- Correa, C., and M. Thiel. 2003b. Mating systems in caridean shrimp (Decapoda: Caridea) and their evolutionary consequences for sexual dimorphism and reproductive biology. *Rev. Chil. Hist. Nat.*, 76: 187– 203.
- Correa, C., Baeza, J. A., Hinojosa, I. A. and Thiel, M. 2003. Male dominance hierarchy and mating tactics in the rock shrimp *Rhynchocinetes typus* (Decapoda: Caridea). *Journal of Crustacean Biology*, 23 (1): 33 – 45.
- Cowan, D. F. 1991. Courtship and Chemical Signals in the American Lobster. Abstracts, Annual Meeting, National Shellfisheries Association, Portland, Maine, USA. p. 284.
- Cowan, D. F. and Atema, J. 1985. Serial monogamy, mate choice and pre- and post-copulatory guarding in lobsters, *Biol. Bull.*, 169: 1 – 550.
- Cowan, D. F. and Atema, J. 1989. Serial monogamy and pheromonal regulation of molting in lobsters, *Homarus americanus*. Eleventh Annual Meeting of the Association for Chemoreception Sciences held at Sarasota, Florida, April 12-16, 1989. Abstracts No. 27 page 692.
- Cowan, D. F. and Atema, J. 1990. Moulting staggering and serial monogamy in American lobster, *H. americanus*. *Anim. Behav.*, 39: 1199 – 1206.
- Cripps, M.C and Nakamura, R.M. 1979. Inhibition of growth of *Macrobrachium rosenbergii* by calcium carbonate water hardness. *Journal of the World Mariculture Society*, 10: 575 – 580.
- Curtis, M. C. and Jones, C. M. 1995. Observations on Monosex culture of Red claw crayfish *Cherax quadricarinatus* von Martens (Decapoda: Parastacidae) in earthen Ponds. *Journal of the World Aquaculture Society*, 26 (2): 154 – 159.

- D'Abramo, L. R., Malecha, S. R., Fuller, M. J. Daniels, W. H. and Heinen, J. M. 1991. Reassessment of the prospects for freshwater prawn culture in the United States: complementary research efforts in Hawaii and Mississippi. In: Sandifer, P. A. (Ed.), *Shrimp Culture in North America and the Caribbean – Advances in World Aquaculture*. World Aquaculture Society, Baton Rouge, LA, USA, pp. 96 – 123.
- Daniels, W. H., Cavalli, R. O. and Smullen, R. P. 2000. Broodstock Management. In: *Freshwater Prawn Culture: The farming of *Macrobrachium rosenbergii**, (Eds. M. B. New and W. C. Valenti), pp. 41 – 51, Blackwell Science Ltd. Oxford, UK.
- Daniels, W. H. and D'Abramo, L. R. 1994. Pond production characteristics of freshwater prawns *Macrobrachium rosenbergii* as influenced by the stocking of size-graded juveniles, *Aquaculture*, 122: 33 – 45.
- Daniels, W. H., D'Abramo, L. R. and Parseval, L. D. 1992. Design and management of a closed, recirculating 'cleanwater' hatchery system for freshwater prawns, *Macrobrachium rosenbergii* De Man. *Journal of Shellfish Research*, 11: 65 – 73.
- Díaz Herrera, F., Sierra Uribe, E., Bückle Ramirez, F. and Garrido Mora, A. 1998. Critical thermal maxima and minima of *Macrobrachium rosenbergii* (Decapoda: Palaemonidae). *J. Therm. Biol.*, 23 (6): 381 – 385.
- Díaz, E. R. and Thiel, M. 2003. Female rock shrimp prefer dominant males. *Journal of Marine Biology Association, UK*. 83: 941 – 942.
- Díaz, E. R. and Thiel, M. 2004. Chemical and Visual Communication During Mate Searching in Rock Shrimp. *Biol. Bull.*, 206: 134 – 143.
- Díaz, G. and Ohno, A. 1986. Possible significance of rearing conditions of ovigerous *Macrobrachium rosenbergii*. In: MacLean, J.L., Dizon, L.B. and Gomez Díaz, G. and Kasahara, S. 1987. The morphological development of *Macrobrachium rosenbergii* larvae. *J. Fac. Appl. Biol. Sci., Hiroshima University*, 26 (1-2): 43 – 56.
- Diesel, R. 1986. Optimal mate searching strategy in the symbiotic spider crab *Inachus phalangium* (Decapoda). *Ethology*, 72: 331 – 328.
- Diesel, R. 1988. Male-female association in the spider crab *Inachus phalangium*: the influence of female reproductive stage and size. *Journal of Crustacean Biology*, 8: 63 – 69.
- Dodson, S.I., Crow, T. A., Peckarsky, B. L., Kats, L. B., Covich, A. P. and Culp, J. M. 1994. Non-visual communication in freshwater benthos—an overview. *Journal of the North America Benthological Society*, 13: 268 – 282.
- Dominey, W. J. 1980. Female mimicry in male bluegill sunfish – a genetic polymorphism? *Nature*, 284 (5756): 546 – 548.

- Dominey, W. J. 1984. Alternative Mating Tactics and Evolutionarily Stable Strategies. *American Zoologist*, 24: 385 – 396.
- Dos Santos, M. J. M. and Valenti, W. C. 2002. Production of Nile Tilapia *Oreochromis niloticus* and freshwater prawn *Macrobrachium rosenbergii* stocked at different densities in polyculture system in Brazil, *Journal of World Aquaculture Society*, 33: 369 – 376.
- Dove, A. D. M. 2005. Ultrastructural features of excretory calcinosis in the lobster, *Homarus americanus* Milne-Edwards. *Journal of Fish Disease*, 28: 313 – 316.
- Dunham, D. W. and Oh, J. W. 1996. Sex discrimination by female *Procambarus clarkii* (Girard, 1852) (Decapoda, Cambaridae): Use of chemical and visual stimuli. *Crustaceana*, 69: 534 – 542.
- Dunham, D. W. and Tierney, A. J. 1983. The communicative cost of crypsis in a hermit crab, *Pagurus marshi*. *Animal Behaviour*, 31: 783 – 787.
- Dunham, P. J. 1978. Sex pheromones in crustacean. *Biol. Rev. Camb. Philos. Soc.*, 53: 555 – 583.
- Dunham, P. J. 1988. Pheromones and behavior in Crustacea. In: *Endocrinology of selected invertebrate types* (Laufer, H. and Downer, R. G. H., eds). New York: Alan R. Liss; 375 – 392.
- Dusenbery, D. B. 1992. *Sensory Ecology: How Organisms Acquire and Responds to Information*. pp. 558. W. H. Freeman, NY. USA.
- Eales, A. J. 1974. Sex pheromone in the shore crab *Carcinus maenas*, and the site of its release from females. *Mar. Behav. Physiol.*, 2: 345 – 355.
- Elwood, R. W., Gibson, J. and Neil, S. 1987. The amorous *Gammarus*: size-assortative mating in *G. pulex*, *Animal Behaviour*, 35: 1 – 6.
- Eisner, T. and Meinwald, J. 1995. *Chemical Ecology*. Washington: National Academic Press.
- Ekerholm, M. and Hallberg, E. 2005. Primer and Short-Range Releaser Pheromone Properties of Premolt Female Urine from the Shore Crab *Carcinus maenas*. *Journal of Chemical Ecology*, 31 (8): 1845 – 1864.
- Elofsson, R. and Hessler, R. R. 2005. The tegumental glands of *Derocheilocaris typica* (Crustacea, Mystacocarida). *Arthropod Structure and Development*, 34: 125 – 138.
- Enquist, M. 1985. Communication during aggressive interactions with particular reference to variation in the choice of behaviour, *Animal Behaviour*, 33: 1152 – 1162.
- FAO. 2008. *Global Aquaculture Production Statistics*. Rome, Italy.

- Figler, M. H., Blank, G. S. and Peeke, H. V. S. 2005. Shelter competition between resident male red swamp crayfish *Procambarus clarkii* (Girard) and conspecific intruders varying by sex and reproductive status. *Mar. Freshw. Behav. Physiol.*, 38: 237 – 248.
- Fincham, A. A. and Wickins, J. F. 1976. Identification of commercial prawns and shrimps. British Museum Publication 779. British Museum, London.
- Fingerman, M. 1987. The endocrine mechanisms of crustaceans. *Journal of Crustacean Biology*, 7: 1 – 24.
- Fingerman, M. 1992. Glands and secretion. In: *Microscopic Anatomy of Invertebrates*. Vol. 10: Decapod Crustacea, (ed. F. W. Harrison and A. G. Humes). pp. 345 – 394. Wiley-Liss, NY.
- Fitzgerald, W. J. and Nelson, S. G. 1979. Development of aquaculture in an island community (Guam, Mariana Islans). *Proc. World Maricult. Soc.*, 10: 39 – 50.
- Flemister, S. C. 1959. Histophysiology of gill and kidney of crab *Ocypode albicans*. *The Biological Bulletin*, 116: 37 – 48.
- Forster, J. R. M. and Wickens, J. F. 1972. Prawn Culture in the United Kingdom. Laboratory Leaflet (New Series), Directorate of Fisheries Research (Great Britain), 27: 32 pp.
- Fujimura, T. and Okamoto, H. 1972. Notes on progress made in developing a mass culturing technique for *Macrobrachium rosenbergii* in Hawaii. In: T.V.R. Pillay (Ed.), *Coastal Aquaculture in the Indo-Pacific Region*. Fishing News Books, Blackwell Science, Oxford, pp. 313 – 327.
- Fuller, E. G., Highison, G. J., Brown, F. and Bayer, C. 1989. Ultrastructure of the crayfish antennal gland revealed by scanning and transmission electron microscopy combined with ultrasonic microdissection. *Journal of Morphology*, 200 (1): 9 – 15.
- Funge-Smith, Simon. J. 1991. Ionic Aspects of the Physiology and Biology of *Macrobrachium rosenbergii* (de Man) 1879. Ph.D. thesis, University of Stirling, Scotland, UK. 220 pp.
- Funk, J. D., Donaldson, E. M. and Dye, H. M. 1973. Induction of precocious sexual development in female pink salmon (*Oncorhynchus gorbuscha*). *Can. J. Zool.*, 51: 493 – 500.
- George, M. J. 1969. Genus *Macrobrachium* (Bate 1868). *Bulletin of the Central Fisheries Research Institute*, 14: 178 – 216.
- Ginsburger-Vogel, T. and Charniaux-Cotton, H. 1982. Sex-determination. In: Abele, L.G. (Ed.), *The Biology of Crustacea*, Vol. 2 Academic Press, New York, NY, pp. 257 – 281.
- Gleeson, R. A. 1980. Pheromone communication in the reproductive behavior of the blue crab, *Callinectes sapidus*. *Mar. Behav. Physiol.*, 7: 119 – 134.

- Gleeson, R. A. 1982. Morphological and behavioural identification of the sensory structures mediating pheromone reception in the blue crab, *Callinectes sapidus*. Biol Bull., 163: 162 – 171.
- Gleeson, R. A. 1991. Intrinsic factors mediating pheromone communication in the blue crab, *Callinectes sapidus*. In: Bauer RT, Martin JW (eds) Crustacean sexual biology. Columbia University Press, New York, p 17 – 32.
- Gleeson, R. A. Adams, M. A. and Smith. A. B. 1984. Characterisation of a sex pheromone in the blue crab *Callinectes sapidus*. J. Chem. Ecol., 10: 913 – 921.
- Goodrich, E. W. 1946. The study of nephridia and genital ducts since 1895 (continued). – Quarterly Journal of Microscopical Science, (4) 86: 303 – 392.
- Goshima, S., Koga, T. and Murai, M. 1996. Mate acceptance and guarding by male fiddler crabs *Uca tetragonon* (Herbst). Journal of Experimental Marine Biology and Ecology, 196: 131 – 143.
- Goshima, S. and Murai, M. 1988. Mating investment of male fiddler crabs, *Uca lacteal*, Animal Behaviour, 36: 1249 – 1251.
- Goshima, S., Kawashima, T. and Wada, S. 1998. Mate choice by male of the hermit crab, *Pagurus filholi*: Do males assess ripeness and / or fecundity of females? Ecological Research, 13: 151 – 161.
- Hardege, J. D., Jennings, A., Hayden, D., Müller, C. T., Pascoe, D., Bentley, M. G. and Clare, A. S. 2002. Novel behavioural assay and partial purification of a female-derived sex pheromone in *Carcinus maenas*, Marine Ecology Progress Series, 244: 179 – 189.
- Hartnoll, R. G. 1969. Mating in the Brachyuran. Crustaceana, 16: 161 – 181.
- Hartnoll, R. G. and Smith, S. M. 1979. Pair Formation in the Edible Crab (Decapoda, Brachyura). Crustaceana, 36 (1): 23 – 28.
- Hasegawa, Y., Hirose, E. and Katakura, Y. 1993. Hormonal control of sexual differentiation and reproduction in Crustacea. American Zoologist, 33: 403 – 411.
- Hazlett, B. A. 1985. Chemical detection of sex and condition in the crayfish. Journal of Chemical Ecology, 11: 181 – 189.
- Heinen, J. M., D'Abramo, L. R., Robinette, H. R. and Murphy, M. J. 1989. Polyculture of two sizes of freshwater prawns, *Macrobrachium rosenbergii*, with fingerlings channel catfish *Ictalurus punctatus*. J. World Aquacult. Soc., 20 (3): 72 – 75.
- Herberholz, J and Schmitz, B. 2001. Signaling via Water Currents in Behavioral Interactions of Snapping Shrimp (*Alpheus heterochaelis*). Biol. Bull., 201: 6 – 16.

- Hessler, R. R. and Elofsson, R. 1991. Excretion system of *Hutchinsoniella macracantha* (Cephalocarida). *Journal of Crustacean Biology*, 11 (3): 356 – 367.
- Hessler, R. R. and Elofsson, R. 2007. An excretory organ in the Mystacocarida (Crustacea). *Arthropod Structure and Development*, 36: 171 – 181.
- Hickling, C.F. 1962. *Fish culture*. Faber and Faber, London, 296 pp.
- Hinojosa, I and Thiel, M. 2003. Somatic and gametic resources in male rock shrimp, *Rhynchocinetes typus*: effect of mating potential and ontogenetic male stage. *Animal Behaviour*, 66: 449 – 458.
- Hinsch, G. W. 1968. Reproductive behaviour in the spider crab, *Libinia emarginate* (L.). *Biological Bulletin*, 135: 272 – 278.
- Holdich, D. M. 2002. *Biology of Freshwater Crayfish*. Blackwell Science Ltd. pp. 702.
- Holliday, C. and Miller, D. 1984. Cellular Mechanisms of Organic Anion Transport in Crustacean Renal Tissue. *Amer. Zool.*, 24: 275 – 284.
- Holthuis, L.B. 1980. FAO species Catalogue. Vol.1. Shrimps and Prawns of the World. An Annotated Catalogue of Species of Interest to Fisheries. FAO Fisheries Synopsis, (125) 1: 271 pp. Rome.
- Holthuis, L.B. 2000. Nomenclature and Taxonomy. In: In New, M.B. and Valenti, W.C. (Eds.). *Freshwater Prawn Culture, The farming of *Macrobrachium rosenbergii**. p: 12-17. Blackwell Science Ltd. Oxford, UK.
- Horner, A. J., Nickles, S. P., Weissburg, M. J. and Derby, C. D. 2006. Source and Specificity of Chemical Cues Mediating Shelter Preference of Caribbean Spiny Lobsters (*Panulirus argus*). *Biol. Bull.*, 211: 128 – 139.
- Hughes, M. 1996. The function of concurrent signals: visual and chemical communication in snapping shrimp. *Anim. Behav.*, 52: 247 – 257.
- Hughes, J. T. and Matthiessen, G. C. 1962. Observations on the biology of the American lobster, *Homarus americanus*. *Limnol. Oceanogr.*, 7: 314 – 321.
- Hulata, G., Karplus, I., Wohlfarth, G. W., Halevy, A., Cohen, D., Sagi, A. and Ra'anan, Z. 1988. The production of *Macrobrachium rosenbergii* in monosex populations. II. Yield characteristics in polyculture ponds. *Bamidgeh*, 40 (1): 9 – 16.
- Hunter, G. A., Donaldson, E. M., Goetz, F. W. and Edgell, P. R. 1982. Production of all female and sterile groups of coho salmon, (*Oncorhynchus kisutch*) and experimental evidence for male heterogamety. *Trans Am. Fish. Soc.*, 111: 367 – 372.
- Hunter, G. A., Solar, I. I., Baker, I. J. and Donaldson, E. M. 1986. Feminization of coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*) by immersion of alevins in solution of estradiol-17 β . *Aquaculture*, 53: 295 – 302.

- Hyatt, G. W. and Salmon, M. 1979. Comparative statistical and information analysis of combat in the fiddler crabs *Uca pugilator* and *U. pugnax*. *Behaviour*, 68: 1 – 23.
- Idyll, C. P. 1971. The crab that shakes hands. *National Geographic*, 139: 254 – 271.
- Itagaki, H. and Thorp, J. H. 1981. Laboratory experiments to determine if crayfish can communicate chemically in a flow-through system. *Journal of Chemical Ecology*, 7 (1): 115 – 126.
- Jackson, S. and Brashers, D. E. 1994. Random factors in ANOVA. Thousand Oaks, CA: Sage Publications. Quantitative Applications in the Social Sciences series no. 98.
- Jackson, N. W. and Elwood, R. W. 1989. How animals make assessments: information gathering by the hermit crab *Pagurus bernhardus*. *Animal Behaviour*, 38: 951 – 957.
- Janssen, J. A. J. 1987. The sex differentiation of the giant freshwater prawn *Macrobrachium rosenbergii*. Network of Aquaculture Centres in Asia, Bangkok, Thailand, Working Paper NACA/WP/87/54, 35 pp.
- Jeng, Ming-Shiou. 1994. Effect of antennular and antennal ablation on pairing behavior of snapping shrimp *Alpheus edwardsii* (Audouin). *Journal of Experimental Marine Biology and Ecology*, 179 (2): 171 – 178.
- Jivoff, P. and Hines, A. 1998. Female behaviour, sexual competition and mate guarding in the blue crab, *Callinectes sapidus*. *Animal Behaviour*, 55: 589 – 603.
- John, C. M. 1957. Bionomics and life history of *Macrobrachium rosenbergii* (De Man). *Bulletin of the Central Research Institute of the University of Kerala*, 15: 93 – 102.
- Johnson, D. S. 1960. Some aspects of the distribution of freshwater priginisms in the Indo-Pacific area and their relevance to the validity of the concept of an orintal region in zoogeography. *Proc. Cent. Bicent. Congr. Biol.*, Singapore, 1958, pp. 170 – 181.
- Johnson, P. T. 1980. Histology of the blue crab, *Callintectes sapidus*- A model for the decapoda. Praege publishers, N.Y. USA. 440 pp.
- Jones, D. R. and Hartnoll, R. G. 1997. Mate selection and mating Behaviour in Spider Crabs. *Estuarine, Coastal and Shelf Science*, 44: 185 – 193.
- Jormalainen, V., Merilaita, S. and Härdling, R. 2000. Dynamics of intersexual conflict over precopulatory mate guarding in two populations of the isopod *Idotea baltica*. *Animal Behaviour*, 60: 85 – 93.
- Juarez, L. M., Holtschmit, K-H., Salmeron, J. J., Smith, M. K. 1987. The effects of chemical and visual communication, space availability, and substratum color on growth of the juvenile freshwater prawn *Macrobrachium rosenbergii* (De

- Man). *Journal of Experimental Marine Biology and Ecology*, 110 (2): 285 – 295.
- Kalinnikova, T.B., Gainutdinov T. M., Seraziev R. K., and Gainutdinov M. K. 2003. The Effect of Ambient Temperature on Intraspecific Interactions Controlling Heat Tolerance in the Oligochaetes *Enchytraeus albidus* and *Tubifex tubifex*. *Russian Journal of Ecology*, 34 (3): 198 – 201.
- Kamiguchi, Y. 1972. Mating behavior in the freshwater prawn, *Palaemon paucidens*. A study of the sex pheromone and its effect on males. *Journal of Faculty of Science, Hokkaido University Series VI, Zoology*, 18 (3): 347 – 355.
- Kamio, M., Araki, M., Nagayama, T., Matsunaga, S. and Fusetani, N. 2005. Behavioral and Electrophysiological Experiments Suggest That the Antennular Outer Flagellum is the Site of Pheromone Reception in the Male Helmet Crab *Telmessus cheiragonus*. *Biol. Bul.*, 208: 12 – 19.
- Kamio, M., S. Matsunaga, and N. Fusetani. 2000. Studies on sex pheromones of the helmet crab, *Telmessus cheiragonus*. 1. An assay based on precopulatory mate-guarding. *Zoo. Sci.*, 17: 731–733.
- Kamio, M., Matsunaga, S. and Fusetani, N. 2003. Observation on the mating behaviour of the helmet crab *Telmessus cheiragonus* (Brachyura: Cheiragonidae). *J. Mar. Biol. Ass., U.K.* 83: 1007 – 1013.
- Karavanich, C. and Atema, J. 1991. Role of Olfaction in Recognition of Dominance in the American Lobster (*Homarus americanus*). *Biol. Bull.*, 181 (2): 359 – 360.
- Karavanich, C. and Atema, J. 1998a. Olfactory recognition of urine signals in dominance fights between male lobster, *Homarus americanus*. *Behaviour*, 135: 719 – 730.
- Karavanich, C. and Atema, J. 1998b. Individual recognition and memory in lobster dominance. *Anim. Behav.*, 56: 1553 – 1560.
- Karlson, P. and Lüscher, M. 1959. 'Pheromones' a new term for a class of biologically active substances. *Nature*, 183: 55 – 56.
- Karnofsky, E. B. and Price, H. J. 1989. Dominance, territoriality and mating in the lobster, *H. americanus*: A mesocosm study. *Mar. Behav. Physiol.*, 15: 101 – 121.
- Karnofsky, E. B., Atema, J., and Elgin, R. H. 1989. Field observations of social behaviour, shelter use and foraging in the lobster, *H. americanus*. *Biol. Bull., (Woods Hole, Mass.)* 176: 239 – 246.
- Karplus, I. 2005. Social control of growth in *Macrobrachium rosenbergii* (De Man): A review and prospects for future research. *Aquaculture research*, 36: 238 – 254.
- Karplus, I., and Hulata, G. 1995. Social control of growth in *Macrobrachium rosenbergii*. V. The effect of unilateral eyestalk ablation on jumpers and laggards. *Aquaculture*, 138: 181 – 190.

- Karplus, I., Barki, A., Israel, Y. and Cohen, S. 1991. Social control of growth in *Macrobrachium rosenbergii*. II. The “leapfrog” growth pattern”. *Aquaculture*, 96: 353 – 365.
- Karplus, I., Hulata, G. and Goldberg, P. 1990. Effect of age at metamorphosis on population structure and production of *Macrobrachium rosenbergii* in polyculture ponds. *Bamidgeh*, 42 (4): 122 – 127.
- Karplus, I., Hulata, G., and Zafirir, S. 1992. Social control of growth in *Macrobrachium rosenbergii*. IV. The mechanism of growth suppression in runts. *Aquaculture*, 106: 275 – 283.
- Karplus, I., Malecha, S. R. and Sagi, A. 2000. The Biology and Management of Size Variation. In: New and Valenti, (Eds.). *Freshwater Prawn Culture, The farming of Macrobrachium rosenbergii*, pp. 259 – 289 Blackwell Science Ltd., Oxford, UK.
- Karplus, I., Hulata, G., Wohlfarth, G. W. and Halevy, A. 1986a. The effect of density of *Macrobrachium rosenbergii* raised in earthen ponds on their population structure and weight distribution. *Aquaculture*, 52: 307 – 320.
- Karplus, I., Hulata, G., Wohlfarth, G. W. and Halevy, A. 1986b. The effect of size grading juvenile *Macrobrachium rosenbergii* prior to stocking on their population structure and production in polyculture. I. Dividing the population into two fractions. *Aquaculture*, 56: 257 – 270.
- Karplus, I., Hulata, G., Wohlfarth, G. W. and Halevy, A. 1987. The effect of size grading juvenile *Macrobrachium rosenbergii* prior to stocking on their population structure and production in polyculture. II. Dividing the population into three fractions. *Aquaculture*, 62: 85 – 95.
- Katakura, Y. 1984. Sex differentiation and androgenic gland hormone in the terrestrial isopod *Armadillium vulgare*. *Symp. Zool. Soc. London*, 53: 127 – 142.
- Katakura, Y. 1989. Endocrine and genetic control of sex differentiation in the Malacostracan Crustacea. *Invertebrate Reproduction and Development*, 16: 177 – 182.
- Kelly, L. S., Snell, T. W. and Lonsdale. 1998. Chemical communication during mating of the harpacticoid *Tigriopus japonicus*. *Phil.Trans. R. Soc. Lond. B*, 353: 737 – 744.
- Khodabandeh, S., Charmantier, G. Blasco, C., Grousset, E. and Charmantier-Daures, M. 2005a. Ontogeny of the antennal glands in the crayfish *Astacus leptodactylus* (Crustacea, Decapoda): anatomical and cell differentiation. *Cell Tissue Res.*, 319: 153 – 165.
- Khodabandeh, S., Kutnik, M., Aujpoulat, F., Charmantier, G. and Charmantier-Daures, M. 2005b. Ontogeny of the antennal glands in the crayfish *Astacus leptodactylus* (Crustacea, Decapoda): immunolocalization of Na⁺ K⁺ -ATPase. *Cell Tissue Res.*, 319: 167 – 174.

- Khodabandeh, S., Charmantier, G. and Charmantier-Daures, M. 2005c. Ultrastructure Studies and Na⁺, K⁺ - ATPase Immunolocalization in the Antennal Urinary Glands of the Lobster *Homarus gammarus* (Crustacea, Decapoda). *Journal of Histochemistry and Cytochemistry*, 53 (10): 1203 – 1214.
- Kirschner, L. B. and Wagner, S. 1965. The site and permeability of the filtration locus in the crayfish antennal gland. *Journal of Experimental Biology*, 43: 385 – 395.
- Kulesh, V.F., and Guiguinyak, Y.G. 1993. Development and growth heterogeneity in oriental river prawn, *Macrobrachium nipponense* (De Haan) (Palaemonidea), in ontogenesis. *Aquaculture and Fisheries management*, 24: 751 – 760.
- Kümmel, G. 1964. Das Cölomsäckchen der Antennendrüse von *Cambarus affinis* Say. (Decapoda, Crustacea) *Zoologische Beiträge*, 10: 227 – 252.
- Kuris, M.A., Ra'anan, Z. Sagi, A., and Cohen, D. 1987. Morphotypic differentiation of male Malaysian giant prawn *Macrobrachium rosenbergii*. *J. Crust. Biol.*, 7 (2): 219 – 237.
- Lam, C. Y. and Wang, J. K. 1986. Engineering research for improvement of freshwater prawn harvest yields in Hawaii. *Aquaculture engineering*, 5: 313 – 323.
- Lam, C. Y. and Wang, J. K. 1987. The effect of harvest frequency on earthen pond prawn production. *Journal of World Aquaculture Society*, 18 (1): 7A (Abstract No.9).
- Lamon, M. S. and Avault, J. W. 1987. Polyculture stocking strategies for channel catfish *Ictalurus punctatus* and the prawn *Macrobrachium rosenbergii*, using one catfish density and three prawn densities with two prawn sizes. *J. World Aquacult. Soc.*, 18 (1): 23A (Abstract No. 83).
- Latruffe, C., McGregor, P. K. and Oliveira. R. F. 1999. Visual signalling and sexual selection in male fiddler crabs *Uca tangeri*. *Mar. Ecol. Prog. Ser.* 189: 233 – 240.
- Laufer, H., Sagi, A. and Ahl, J. S. B. 1992. Juvenile hormone-like compounds in Crustacea and their implications for reproductive behavior. *Advances in regulation of insect reproduction*, B. Bennettova, I. Gelbic and T. Soldan, (Eds.). Institute of Entomology, Czech Acad. Sci., pp. 153 – 155.
- Laufer, H., Sagi, A., Ahl, J. S. B., and Homola, E. 1992. Methylfamesoate appears to be crustacean reproductive hormone. *Invertebrate Reproduction and Development*, 22: 17 – 19.
- Law, A. T., Wong, Y. H. and Abol-Munafi, A. B. 2002. Effect of hydrogen ion on *Macrobrachium rosenbergii* egg hatchability in brackish water. *Aquaculture*, 214: 247 – 251.
- Lee, C. L. and Fielder, D. R. 1982. Maintenance and Reproductive Behaviour in the Freshwater Prawn *Macrobrachium australiense* Holthuis (Crustacea : Decapoda : Palaemonidae). *Aust. J. Mar. Freshwater Res.*, 33: 629 – 646.

- Lee, C. L. and Fielder, D. R. 1983. Post larval growth of the freshwater prawn, *Macrobrachium australiense* Holthuis, 1950 (Crustacea: Decapoda: Palaemonidae). *Aquaculture*, 30: 63 – 70.
- Lee, T-H., Shigesawa, R. and Yamazaki, F., 1993. Partial masculinization of female *Eriochei japonicus* (Brachyura, Grapsidae) by androgenic gland implantation. *Suisanzoshku*, 41 (3): 311 – 319. (Japanese with English Abstract).
- Leeuwan, H. C. van and Maly, E. J. 1991. Changes in swimming behavior of male *Diaptomus leptopus* (Copepoda: Calanoida) in response to gravid females. *Limnol. Oceanogr.*, 36 (6): 118 – 1195.
- Legrand, J.J., Legrand-Hamelin, E. and Juchault, P. 1987. Sex determination in crustacea. *Biol. Rev.*, 62: 439 – 470.
- Leinders-Zufall, T., Lane, A. P., Puche, A. C., Ma, W., Novotny, M. V., Shipley, M. T. and Zufall, F. 2000. Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature*, 405: 792 – 796.
- Li, W., Sorensen, P. W. and Gallaher, D. D. 1995. The olfactory system of migratory adult sea lamprey is specifically and acutely sensitive to unique bile acids released by conspecific larvae. *Journal of General Physiology*, 105: 569-587.
- Liao, I-C. and Chao, N-H. 1982. Progress of *Macrobrachium rosenbergii* farming and its extension in Taiwan. In: M. B. New (Ed.), *Giant Prawn Farming, Developments in Aquaculture and Fisheries Science*, Vol. 10. Elsevier, Amsterdam, pp. 357 – 379.
- Lin, S. C., Liou, C. H. and Cheng, J. H. 2000. The role of the antennal glands in ion and body volume regulation of cannulated *Penaeus monodon* reared in various salinity conditions. *Comparative Biochemistry and physiology, Part A*. 127: 121 – 129.
- Ling, S. W. 1969. The general biology and development of *Macrobrachium rosenbergii* (de Man). *FAO Fisheries Report*, 57 (3): 589 – 606.
- Ling, S. W. and Costello, T. J. 1979. The culture of freshwater prawns: a review. In *Advances in Aquaculture*, T.V.R. Pillay and W.A. Dill (editors), Fishing News Books Ltd. Farnham, England.
- Linn, C. E., Campbell, M. G. and Roelofs, W. L. 1988. Temperature modulation of behavioural thresholds controlling male moth sex pheromone response specificity. *Physiological Entomology*, 13 (1): 59 – 67.
- Malecha, S. R. 1977. Genetic and selective breeding of *M. rosenbergii*. In: *Shrimp and Prawn Farming in the Western Hemisphere* (Hanson, J.A. and Goodwin, H.L. Eds.), pp. 328 – 355. Dowden, Hutchinson and Ross, Stroudsburg, Pennsylvania, USA.
- Malecha, S. R. 1983a. Commercial pond production of the freshwater prawn *Macrobrachium rosenbergii*. In: *CRC Handbook of Marine Science Vol. I*,

- Aquaculture of Crustaceans, ed. J. P. McVey, CRC Press, Boca Raton, Florida, USA, pp. 231 – 259.
- Malecha, S. R. 1983b. Commercial seed production of the freshwater prawn *Macrobrachium rosenbergii*. In: CRC Handbook of Marine Science Vol. I, Aquaculture of Crustaceans, ed. J. P. McVey, CRC Press, Boca Raton, Florida, USA, pp. 205 – 230.
- Malecha, S. R. 1986. New techniques for the Assessment and Optimal Management of Growth and Standing Crop Variation in the Cultured Freshwater Prawn, *Macrobrachium rosenbergii*. Aquaculture Engineering, 5: 183 – 197.
- Malecha, S. R., Barck, L. and Masuno, S. 1987. A new stock management system for year round culture of the freshwater prawn, *Macrobrachium rosenbergii*. Journal of World Aquaculture Society, 18 (1): 33A (Abstract No. 144).
- Malecha, S. R., Masuno, S. and Onizuka, D. 1984. The feasibility of measuring the heritability of growth pattern variation in juvenile freshwater prawns, *Macrobrachium rosenbergii* (De Man). Aquaculture, 38: 347 – 363.
- Malecha, S. R., Nevin, P. A., Ha, P., Barck, L. E., Lamadrid-Rose, Y. Masuno, S. and Hedgecock, D. 1992. Sex-ratio and sex-determination in progeny from crosses of surgically sex-reversed freshwater prawn, *Macrobrachium rosenbergii*. Aquaculture, 105: 201 – 218.
- Malecha, S.R., Sarver, D. and Onizuka, D. 1980. Approaches to the study of domestication in the freshwater prawn, *M. rosenbergii*, with special emphasis on the Anuenue and Malaysian stocks. Proc. World. Maricult. Soc., 11: 500 – 528.
- Maluf, N. S. R. 1939. On the anatomy of the kidney of the crayfish and on the absorption of chloride from freshwater by this animal. Zoologische Jahrbücher, Abteilung Allgemeine Zoologie, 59: 515 – 534.
- Mantel, L. H. and Farmer, L. L. 1983. Osmotic and ionic regulation. In: Mantel, L. H. (Ed.), The Biology of Crustacea. Internal Anatomy and Physiological Regulation, Vol. 5. Academic Press, New York, pp. 53 – 161.
- Mantel, S. K. and Dudgeon, D. 2005. Reproduction and Sexual Dimorphism of the Palaemonid shrimp *Macrobrachium hainanense* in Hong Kong Streams. Journal of Crustacean Biology, 25 (3): 450 – 459.
- Marshall, J., T. W. Cronin, N. Shashar, and M. Land. 1999. Behavioural evidence for polarisation vision in stomatopods reveals a potential channel for communication. Curr. Biol., 9: 755 – 758.
- Mathews, L. M. 2003. Tests of the mate-guarding hypothesis for social monogamy: male snapping shrimp prefer to associate with high-value females. Behavioral Ecology, 14 (1): 63 – 67.
- McLaughlin, P. A. 1979. Comparative Morphology of Recent Crustacea. W.H. Freeman and Company, San Francisco, pp 1-177.

- McLeese, D. W. 1970. Detection of dissolved substances by the American lobster (*Homarus americanus*) and olfactory attraction between lobsters. J. Fish. Res. Board Can., 27: 1371 – 1378.
- McLeese, D. W. 1973. Chemical communication among lobster (*Homarus americanus*). J. Fish. Res. Board Can., 30: 775 – 778.
- McLeese, D. W., Spraggins, R. L., Bose, A. K. and Praminik, B. N. 1977. Chemical and behavioural studies of the sex attractant of the lobster (*Homarus americanus*). Marine Behaviour and Physiology, 4: 219 – 232.
- McPhie, D. and Atema, J. 1984. Chemical communication in lobster: Information currents. Biol. Bull., 167: 510 – 511.
- Meusy, J. J. and Payen, G. G. 1988. Female reproduction in malacostracan Crustacea. Zool. Sci., 5: 217 – 265.
- Moore, P. A. and Bergman, D., A. 2005. The Smell of Success and Failure: the Role of Intrinsic and Extrinsic Chemical Signals on the Social Behavior of Crayfish. Integr. Comp. Biol., 45:650 – 657.
- Murai, M., Goshima, S. Kawai, K. and Yong, Hoi-Sen.1996. Pair formation in the burrows of the fiddler crab *Uca rosea* (Decapoda: Ocypodidae). Journal of Crustacean Biology, 16 (3): 522 – 528.
- Nagabhushanam, R., Machale, P. R. and Sarojini, R. 1989. Pheromonal and hormonal control of reproduction in the freshwater prawn, *Macrobrachium kistnensis*. Proc. Indian Acad. Sci. (Anim. Sci.), 98 (2): 95 – 98.
- Nagamine, C. M., and Knight, A. W. 1980. Development, maturation, and function of some sexually dimorphic structures of the Malaysian prawn, *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae). Crustaceana, 39 (2): 141 – 152.
- Nagamine, C. M., and Knight, A. W. 1987. Masculinization of female crayfish, *Procambarus clarki* (Girard). International Journal of Invertebrate and Reproduction, 11: 77 – 87.
- Nagamine, C., Knight, A. W., Maggenti, A. and Paxman, G. 1980a. Effects of androgenic gland ablation on male primary and secondary sexual characteristic in the Malaysian prawn, *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae) with first evidence of induced feminization in a nonhermaphroditic decapod. General and Comparative Endocrinology, 41: 423 – 441.
- Nagamine, C. M., Knight, A. W., Maggenti, A. and Paxman, G. 1980b. Masculinization of Female *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae) by Androgenic Gland Implantation. General and Comparative Endocrinology, 41: 442 – 457.

- Nakamura, K and Nishigaki, Y. 1991. Structure of antennal gland in Kuruma prawn *Penaeus japonicus*. Nippon Suisan Gakkaishi/Bull. Jap. Soc. Sci. Fish., 57 (10): 1859 – 1863.
- Nakasone, Y. and Murai, M. 1998. Mating Behavior *Uca lacteal perplexa* (Decapoda: Ocypodidae). Journal of Crustacean Biology, 18 (1): 70 – 77.
- Nelson, K and Hedgecock, D. 1983. Size-dependence of growth inhibition among juvenile lobsters (*Homarus*). J. Exp. Mar. Biol. Ecol., 66: 125 – 134.
- Nelson, K., Hedgecock, D., Borgeston, W., Johnson, E., Daggett, R., and Aronstein, D. 1980. Density-dependent growth inhibition in lobsters, *Homarus* (Decapoda, Nephropidae). Biol. Bull., 159: 162 – 176.
- New, M. B. 1990. Freshwater prawn culture: a review. Aquaculture, 88: 99 – 143.
- New, M. B. 1995. Status of freshwater farming: a review. Aquaculture Research, 26: 1 – 54.
- New, M. B. 2000. History and global status of freshwater prawn farming. In: Freshwater Prawn Culture, The Farming of *Macrobrachium rosenbergii*. (Eds. New, M. B. and Valenti, W. C.), p: 1 – 11, Blackwell Science Ltd., Oxford, UK.
- New, M. B. 2005. Freshwater prawn farming: global status, recent research and a glance at the future. Aquaculture Research, 36: 210 – 230.
- New, M. B. and Singholka, S. 1985. Freshwater prawn farming. FAO Fisheries Technical Paper, 225 FIRI/T225. Rome, Italy.
- Nishida, R. Schulz, S., Kim, C. S., Fukami, H., Kuwahara, Y., Honda, K. and Hayashi, N. 1996. Male sex pheromone of a giant danaine butterfly, *Idea leucinoe*. J. Chem. Ecol., 22: 949 – 972.
- Niu, C., Lee, D., Goshima, S. and Nakao, S. 2003. Effects of temperature on food consumption, growth, and Oxygen consumption of freshwater prawn *Macrobrachium rosenbergii* (de Man 1879) post-larvae. Aquaculture Research, 34: 501 – 506.
- Ohs, C. L., D'Abramo, L. R. and Kelly, A. M. 2006. Effect of Dietary Administration of 17 α -methyltestosterone on the Sex Ratio of Postlarval Freshwater Prawn, *Macrobrachium rosenbergii*, During the Nursery Stage of Culture. Journal of The World Aquaculture Society, 37 (3): 328 – 333.
- Ono, Tomohiro. 1992. Effect of rearing temperature on pheromone component ratio in potato tuberworm moth, *Phthorimaea operculella*, (Lepidoptera: Gelechiidae). Journal of Chemical Ecology, 19 (1): 71 – 81.
- Panikkar, N. K., 1941. Osmoregulation in some palaemonid prawns. J. mar. biol. Ass. U.K., 25: 317 – 59.

- Parnes, S., Raviv, S. Shechter, A. and Sagi, A. 2006. Males also have their time of the month! Cyclic disposal of old spermatophores, timed by the molt cycle, in a marine shrimp. *The Journal of Experimental Biology*, 209: 4974 – 4983.
- Patil, P. G. and Krishnan, M. 1997. The social impacts of shrimp farming in Nellore district, India. *Aquaculture Asia*, 2: 3 – 5.
- Peebles, J. B. 1980. Competition and habitat partitioning by the giant freshwater prawn *Macrobrachium rosenbergii* (de Man) (Decapoda, Palamonidae). *Crustaceana*, 38 (1): 49 – 54.
- Percy-Cunningham, J. E. and MacDonald, J. A., 1987. Biology and ultrastructure of sex pheromone-producing glands. In: Prestwich, G. D. and Blomquist, G. J., Eds, *Pheromone Biochemistry*, Academic Press, Orlando, FL, pp. 27–75.
- Perdue, J A. and Nakamura, R. 1976. The effect of salinity on the growth of *Macrobrachium rosenbergii*. *Proc. World Maric. Soc.*, 7: 647 – 654.
- Peterson, D. R. and Loizzi, R. F. 1973. Regional cytology and cytochemistry of the crayfish kidney tubule. *Journal of Morphology*, 141: 133 – 146.
- Peterson, D. R. and Loizzi, R. F. 1974. Ultrastructure of the Crayfish Kidney–Coelomosac, Labyrinth, Nephridial Canal. *Journal of Morphology*, 142: 241 – 264.
- Pierce, B. A. and Law, E. A. 1982. Applications of a new radiotracer method for investigating prawn (*Macrobrachium rosenbergii*) ecology and digestive physiology. *Proc. World Maricult. Soc.*, 13: 28 – 40.
- Polkinghorne, C. N., Olson, J. M., Gallaher, D. G. and Sorensen, P. W. 2001. Larval sea lamprey release two unique bile acids to the water at a rate sufficient to produce detectable riverine pheromone plumes. *Fish Physiology and Biochemistry*, 24 (1): 15 – 30.
- Ra'anan, Z. 1983. The effect of size ranking on the moulting cycle of juvenile stages of the freshwater prawn *Macrobrachium rosenbergii* (de Man) when reared individually and in pairs (Decapoda, Caridea). *Crustaceana*, 45 (2): 131 – 138. E. J. Brill, Leiden.
- Ra'anan, Z., and Cohen, D. 1983. Production of the freshwater prawn *Macrobrachium rosenbergii* in Israel. II. Selective stocking of size subpopulation. *Aquaculture*, 31: 369 – 379.
- Ra'anan, Z., and Cohen, D. 1984a. Characterization of size distribution development in the freshwater prawn *Macrobrachium rosenbergii* (de Man) juvenile populations. *Crustaceana*, 46: 271 – 282.
- Ra'anan, Z., and Cohen, D. 1984b. The effect of group interactions on the development of size distribution in *Macrobrachium rosenbergii* (de Man) juvenile populations. *Biol. Bull.*, 166: 22 – 31.

- Ra'anan, Z., and Cohen, D. 1985. Ontogeny of social structure and population dynamic in the giant freshwater prawn *Macrobrachium rosenbergii* (de Man). In "Crustacean Issues 3: Crustacean Growth: Factors in adult Growth," (Adrian Wenner, Ed.), pp. 227 – 311. A. A. Balkema Publishers.
- Ra'anan, Z., and Sagi, A. 1985. Alternative mating strategies in male morphotypes of the freshwater prawn *Macrobrachium rosenbergii* (de Man). Biol. Bull., 169: 592 – 601.
- Ra'anan, Z., Sagi, A., Wax, Y., Karplus, I., Hulata, G. and Kuris, A. 1991. Growth, size rank, and maturation of the freshwater prawn, *Macrobrachium rosenbergii*: analysis of marked prawns in an experimental population. Biol. Bull., 181: 379 – 386.
- Raethke, N., MacDiarmid, A. B. and Montgomery, J. C. 2004. The role of olfaction during mating in the southern temperate spiny lobster *Jasus edwardsii*. Hormones and Behavior, 46: 311 – 318.
- Rahman, N., Dunham, D. W. and Govind, C. K. 2001. Mate recognition and pairing in the big-clawed snapping shrimp *Alpheus heterochelis*. Mar. Freshw. Behav. Physiol., 34: 213 – 226.
- Raina, A. K. 2003. Pheromone production in corn earworm: effect of temperature and humidity. Southwestern Entomologist, 28 (2): 115 – 120.
- Ramesh, G. 2001. Tips for successful freshwater prawn culture. SEAFDEC, Asian Aquaculture Vol. XXIII Nos. 1 – 2 January – April.
- Ranjeet, K. and Kurup, B. M. 2002. Management strategies associating batch-graded and size-graded post-larvae can reduce heterogeneous individual growth in *Macrobrachium rosenbergii* (de Man). Aquaculture Research, 33: 1221 – 1231.
- Rao, K. J. 1991. Reproductive biology of the giant freshwater prawn *Macrobrachium rosenbergii* (De Man) from Lake Kolleru (Andhra Pradesh). Indian Journal of Animal Science, 61: 780 – 787.
- Rao, R. M. 1967. Studies on the biology of *Macrobrachium rosenbergii* (de Man) of the Hooghly estuary with notes on its fishery. Proc. Nat. Inst. Sci. India, Part B. 33: 252 – 279.
- Ratchford, S. G. and Eggleston, D. B. 2000. Temporal shift in the presence of a chemical cue contributes to a diel shift in sociality. Anim. Behav., 59: 793 – 799.
- Reid, D. G., Abello, P., Warman, C. G. and Naylor, E. 1994. Size-related mating success in the shore crabs *Carcinus maenas* (Crustacea: Brachyura). Journal of Zoology, London, 232: 397 – 407.
- Reynolds, J. and Gross, M. R. 1990. Costs and benefits of female mates choice: Is there a lek paradox? Am. Nat., 136: 230 – 243.

- Rico-Martínez, R. and Snell, T.W. 1997. Comparative binding of antibody to a mate recognition pheromone on female *Brachionus plicatilis* and *Brachionus rotundiformis* (Rotifera). *Hydrobiologia*, 358 (1 – 3): 71 – 76.
- Riegel, J. A. 1963. Micropuncture studies of chloride volume and osmotic pressure in the crayfish antennal gland. *Journal of Experimental Biology*, 40: 487 – 492.
- Riegel, J. A. 1965. Micropuncture studies of volumes of Sodium, Potassium and Inulin in the crayfish antennal gland. *Journal of Experimental Biology*, 42: 379 – 384.
- Riegel, J. A. 1966. Analysis of formed bodies in urine removed from the crayfish antennal gland by micropuncture. *Journal of Experimental Biology*, 44: 387 – 395.
- Rinderhagen, M., Ritterhoff, J. and Zauke, Gerd-Peter. 2000. Crustaceans as Bioindicators. In: *Biomonitoring of Polluted Water—Reviews on Actual Topics* (A. Gerhardt, ed.), Trans Tech Publications – Scitech Publications, Environmental Research Forum, 9: 161 – 194.
- Rittschof, D. 1992. Chemosensation in the daily life of crabs. *Amer. Zool.*, 32: 363 – 369.
- Roelofs, W. L., Hill, A. S., Linn, C. E., Meinwald, J., Jain, S. C., Herbert, H. J., and Smith, R. F. 1982. Sex pheromone of the winter moth, a geometrid with unusually low temperature *Precopulatory Responses*. *Science*, 217 (4560): 657 – 659.
- Rogers, J. V. and Wheatly, M. G. 1997. Accumulation of calcium in the antennal gland during the molting cycle of the freshwater crayfish *Procambarus clarkii*. *Invertebrate Biology*, 116 (3): 248 – 254.
- Rosenthal, G. G. and Lobel, P. S. 2006. Communication. In: *Behaviour and Physiology of Fish: VOL 24*. (Eds: Sloman, K. A., Wilson, R. W. and Balshine, S.) pp 39 – 78. Academic Press, San Diego, California, USA.
- Rubenstein, D. and Hazlett, B. 1974. Examination of the agonistic behaviour of the crayfish *Orconectes virilise* by character analysis. *Behaviour*, 50: 193 – 216.
- Ruello, N. V., Moffitt, P. F. and Phillips, S. G. 1973. Reproductive Behaviour In Captive Freshwater Shrimp *Macrobrachium Australiense* Holthuis. *Aust. J. Mar. Freshwater Res.*, 24: 197 – 202.
- Rungsin, W. Paankhao, N. and Na-Nakorn, U. 2006. Production of all-male stock by neofemale technology of the Thai strain of freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture*, 259: 88 – 94.
- Ryan, E. D. 1966. Pheromone: evidence in a decapod crustacean. *Science*, 151, No. 3708: 340 – 341.
- Sagi, A., and Ra'anana, Z. 1988. Morphotypic differentiation of the freshwater prawn *Macrobrachium rosenbergii* males: Change in the midgut glands and the reproductive system. *J. Crustacean Biol.*, 8 (1): 43 – 47.

- Sagi, A., Milner, Y. and Cohen, D. 1988. Spermatogenesis and sperm storage in the testes of the behaviorally distinctive male morphotypes of *Macrobrachium rosenbergii* (Decapoda, Palaemonidae). *Bioll. Bull.*, 174: 330 – 336.
- Sagi, A., Ra'anan, Z., Cohen, D., and Wax, Y. 1986. Production of *M. rosenbergii* monosex populations: Yield characteristics under intensive monoculture conditions in cages. *Aquaculture*, 51: 265 – 275.
- Sainte-Marie, B., Sévigny, J. M. and Gauthier, Y. 1997. Laboratory behavior of adolescent and adult males of the snow crab (*Chionoecetes opilio*) (Brachyura: Majidae) mated noncompetitively and competitively with primiparous females. *Canadian Journal of Fisheries and aquatic Science*, 54: 239 – 248.
- Salmon, M. 1983. Courtship mating system and sexual selection in decapods. pp. 143 – 169. In: S. Rebach and D. Dunham, (Eds.) *Studies in Adaptations*. John Wiley and Sons, New York.
- Sandifer, P. A., and Smith, T. I. 1975. Effects of population density on growth and survival of *Macrobrachium rosenbergii* reared in recalculation water management systems. *Proceeding of the World Mariculture Society*, 6: 43 – 53.
- Sandifer, P. A., and Smith, T. I. 1979. Possible significance of variation in the larval development of the palaemonid shrimp. *J. Exp. Mar. Biol. Ecol.*, 39: 55 – 64.
- Sandifer, P. A., and Smith, T. I. 1985. Freshwater Prawns. In: Huner, J. V., and Brown, E. E., (Eds.), *Crustacean and Mollusk Aquaculture in the United State of America*. AVI Publishing Co. Inc., Westport, Connecticut, USA, pp. 63 – 125.
- Sandifer, P. A., Hopkins, J. S., and Smith, T. I. J. 1975. Observations on salinity tolerance and osmoregulation in laboratory-reared *Macrobrachium rosenbergii* post-larvae (Crustacea: Cardiea). *Aquaculture*, 6: 103 – 114.
- Sandifer, P. A., Hopkins, J. S., and Smith, T. I. J. 1977. Status of *Macrobrachium rosenbergii* hatcheries, 1976. In: *Shrimp and Prawn Farming in the Western Hemisphere*, J. A. Hanson and H.L. Goodwin (Eds.), Dowden, Hutchinson, and Ross, Inc. Stroudsburg, Pennsylvania.
- Sarojini, S. 1962. The androgenic organ in some Indian Crustacea II. Effect of its implantation into the female in *Ocypoda platytarsis*, Milne Edwards. *J. Zool. Soc. India*, 14: 141 – 144.
- Sarojini, R., Nagabuhushanam, R., and Fingerman, M. 1995. In vivo effects of dopamine and dopaminergic antagonists on testicular maturation in the red swamp crayfish, *Procambarus clarkii*. *Biol. Bull.*, 189: 340 – 346.
- Schaffner, A. and Rodewald, R. 1978. Filtration Barriers in the Coelomicsac of the Crayfish, *Procambarus clarkia*. *Journal of Ultrastructure Research*, 65: 36 – 47.
- Schmidt-Nielsen, B., Gertz, K. H. and Davis, L. E. 1968. Excretion and ultrastructure of the antennal gland of the fiddler crab *Uca mordax*. *J. Morph.*, 125 (4): 473 – 496.

- Schmitt, B. C. and Ache, B. W. 1979. Olfaction: responses of a decapod crustacean are enhanced by flicking. *Science*, NY 205: 204 – 206.
- Schone, H. 1961. Complex behaviour. In: *The Physiology of Crustacea.*, T. H. Waterman (Ed.), Vol. 2 pp. 465 – 520. Academic Press: New York.
- Segal, E. and Roe, A. 1975. Growth and behavior of post-juvenile *Macrobrachium rosenbergii*. (De Man) in close confinement. *Journal of the World Mariculture Society*, 6: 67 – 88.
- Seifert, P. 1982. Studies on the sex pheromone of the shore crab, *Carcinus maenas*, with special regard to ecdysone excretion. *Ophelia*, 21: 147 – 158.
- Shivers, R. R. and Chauvin, W. J. 1977. Intercellular junctions of antennal gland epithelial cells in the crayfish, *Orconectes virilis*, *Cell and Tissue Research*, 175 (4): 425 – 428.
- Shorey, H. H. 1976. *Animal Communication by Pheromones*, Academic Press, New York. 176 pp.
- Shuang-lin, Z. and Nai-cheng, J. 2007. Microstructure and ultrastructure of antennal gland of *Penaeus monodon*. *Journal of Zhejiang University (Science Edition)*, 34: 91 – 95.
- Shuster, S. M. and Sassaman, C. 1997. Genetic interaction between male mating strategy and sex ratio in marine isopod. *Nature*, 388: 373 – 377.
- Shuster, S., 1987. Alternative reproductive behaviour: three discrete male morphs in *Paracerceis sculpta*, an intertidal isopod from the northern Gulf of California. *J. Crustacean Biol.*, 7: 318 – 327.
- Siddiqui, A. Q., Al-Hinty, H. M. and Ali, S. A. 1996. Evaluation of the production potential of *Macrobrachium rosenbergii* (de Man) in monoculture and Polyculture with Nile tilapia and common carp in Saudi Arabia. *Aquaculture Research*, 27: 515 – 521.
- Siddiqui, A. Q., Al-Hafedh, Y.S., Al-Harbi, A.H. and Ali, A.A. 1997. Effects of stocking density and monosex culture of freshwater prawn *Macrobrachium rosenbergii* on growth and production in concrete tanks in Saudi Arabia. *Journal of the World Aquaculture Society*, 28, (1): 106 – 111.
- Simon, Jodie L. and Moore, Paul A. 2007. Male–Female Communication in the Crayfish *Orconectes rusticus*: The Use of Urinary Signals in Reproductive and Non-Reproductive Pairings. *Ethnology*, 113: 740 – 754.
- Smith, T. I. J., Sandifer, P. A. and Smith, H. H. 1978. Population structure of Malaysian prawns, *Macrobrachium rosenbergii* (de Man), reared in earthen ponds in South Carolina, 1974. *Journal of the World Mariculture Society*, 9: 21 – 38.
- Smith, T. I. J., Waltz, W. and Sandifer, P. A. 1980. Processing yields for Malaysian prawns and the implications. *Proceeding of the World Mariculture Society*, 11: 557 – 569.

- Snedden, W. A. 1990. Determinants of male mating success in the temperate crayfish *Orconectes rusticus*: chela size and sperm competition. *Behaviour*, 115: 100 – 113.
- Sneddon, L. U., Huntingford, F. A., Taylor, A. C. and Clare, A. S. 2003. Female Sex Pheromone-Mediated Effects on Behavior and Consequences of Male Competition in the Shore Crab (*Carcinus maenas*). *Journal of Chemical Ecology*, 29 (1): 55 – 70.
- Sneddon, L. U., Taylor, A. C. Huntingford, F. A., Watson, D. G. 2000. Agonistic Behaviour and Biogenic Amines in Shore Crabs, *Carcinus maenas*. *The Journal of Experimental Biology*, 203: 537 – 545.
- Solon, M. H. and Cobb, J. S. 1980. Antennae-whipping behaviour in the American lobster, *H. americanus* (Milne Edwards). *J. Mar. Biol. Ecol.*, 157: 3 – 18.
- Sorensen, P. W. and Stacey, N. E. 2004. Brief review of fish pheromones and discussion of their possible uses in the control of non-indigenous teleost fishes. *New Zealand Journal of Marine and Freshwater Research*, 38: 399 – 417.
- Stebbing, P. D., Bentley, M. G. and Watson, G. J. 2003. Mating behaviour and evidence for a female released courtship pheromone in the signal crayfish *Pacifastacus leniusculus*. *J. chem. Ecol.*, 29: 465 – 475.
- Steenken, B. and Schminke, H. K. 1996. Ultrastructure of Maxillary Gland of *Antrobathynella stammeri* (Syncarida, Malacostraca). *Journal of Morphology*, 228: 107 – 117.
- Suzuki, S. and Yaamsaki, K. 1997. Sexual bipotentiality of developing ovaries in the Terrestrial Isopod *Armadillidium vulgare* (Malacostraca, Crustacea). *General and Comparative Endocrinology*, 107: 136 – 146.
- Tanaka, K. 2003. Population dynamics of the sponge-dwelling gnathiid isopod *Elaphognathia cornigera*. *The Journal of the Marine Biological Association of the United Kingdom*, 83: 95 – 102.
- Taylor, P. M. and Harris, R. R. 1986. Osmoregulation in *Corophium curispinum* (Crustacea: Amphipoda) a recent coloniser of freshwater. *Journal of Comparative Physiology, B*, 156: 331 – 337.
- Telecky, T. M. 1984. Alternative Male Reproductive Strategies in the Giant Malaysian Prawn, *Macrobrachium rosenbergii*. *Pacific Science*, 38: 372 – 373.
- Templeman, W. 1934. Mating in the American lobster, *Contrib. Can. Biol. Fish.*, (N. S.) 8: 423 – 458.
- Templeman, W. 1936. Further contributions to mating in the American lobster. *J. Biol. Board Can.*, 2: 223 – 226.
- Thampy, D.M. and John, P.A. 1973. Observations on variation in the male sex characters and their relation to the androgenic gland in the shrimp *Macrobrachium idea* (Heller). *Acta. Zool.*, 54: 193 – 200.

- Thomas, B. 1998. An investigation of putative pheromone mediated behaviour in *Macrobrachium rosenbergii* (de Man, 1879). M.Sc. Thesis, University of Stirling, Stirling, UK.
- Thomas, C. W., Carter, C. G. and Crear, B. J. 2003. Feed availability and its relationship to survival, growth, dominance and the agonistic behaviour of the southern rock lobster, *Jasus edwardsii* in captivity. *Aquaculture*, 215: 45 – 65.
- Thomas, F. Liautard, C. Cézilly, F. and Renaud, F. 1998. A finite time horizon influences sequential mate choice in male *Gammarus aequicauda* (Amphipoda). *Canadian Journal of Zoology*, 76: 400 – 404.
- Thornhill, R. and Alcock, J. 1983. *The Evolution of Insect Mating Systems*. Harvard University Press, Cambridge, Massachusetts, USA.
- Tidwell, J. H., Coyle, S. D., Bright, L. A. Van Arnum, A. and Weible, C. 2003. The effects of size grading and length of nursery period on growth and population structure of freshwater prawns stocked in temperate zone ponds with added substrates. *Aquaculture*, 218: 209 – 218.
- Tidwell, J. H., D'Abramo, L. R., Webster, C. D., Coyle, S. D. and Daniels, W. H. 1996. A standardized comparison of semi-intensive pond culture of freshwater prawns *Macrobrachium rosenbergii* at different latitudes: production increase associated with lower water temperatures. *Aquaculture*, 141: 145 – 158.
- Tidwell, J. H., Webster, C. D. Goodgame-Tiu, L. and D'Abramo, L. R. 1994. Population characteristics of *Macrobrachium rosenbergii* fed diets containing different protein sources under coolwater conditions in earthen ponds. *Aquaculture*, 126 (3-4): 271 – 281.
- Tinbergen, N. 1951. *The Study of Instinct*. Oxford University Press, Oxford, 250 pages.
- Tombes, A. S. and Foster, M. W. 1979. Growth of appendix masculina and appendix interna in juvenile *Macrobrachium rosenbergii* (de Man) (Decapoda, Caridea) *Crustaceana*, Supp., 5: 179 – 184.
- Tunsutapanich, A., Chalaypote, S. and Phuhoung, P. 1982. *Macrobrachium* farming in areas with irregular water supplies. In: M. B. New (Ed.). *Giant Prawn Farming, Developments in Aquaculture and Fisheries Science*, Vol. 10. Elsevier, Amsterdam, pp. 207 – 212.
- Tyson, G. E. 1969. The fine structure of the maxillary gland of the brine shrimp, *Artemia salina*: The efferent Duct. *Z. Zellforsch*, 93: 151 – 163.
- Ueno, M., Inoue, Y. and Niwa, N. 1997. Podocytes of the freshwater shrimp – fine structure and effect of injected trypan blue. *Japanese Society of Electron Microscopy, Journal of Electron Microscopy*, 46 (6): 585 – 490.
- Uno, Y. and Kwon, C. S. 1969. Larval development of *Macrobrachium rosenbergii* (de Man) reared in the laboratory. *Journal of the Tokyo University of Fisheries*, 55: 179 – 190.

- Vasquez, O. E., Rouse, D. B. and Rogers, W. A. 1989. Growth response of *Macrobrachium rosenbergii* to different levels of hardness. *Journal of the World Aquaculture Society*, 20, (2): 90 – 92.
- Vogt, G. 2002. Functional Anatomy. In: *Biology of Freshwater Crayfish*, Holdich, D. M. (Ed.), pp. 53 – 151. Blackwell Science Ltd. Oxford, UK.
- Waddy, S. L. and Aiken, D. E. 1990. Intermolt insemination, an alternative mating strategy for the American lobster (*H. americanus*). *Can. J. Fish. Aquat. Sci.*, 47: 2402 – 2406.
- Walter, U. and Wägele, J. W. 1990. Ultrastructure of the Maxillary Gland of *Asellus aquaticus* (Crustacea, Isopoda). *Journal of Morphology*, 204: 281 – 293.
- Ward, P. I. 1983. The effect of size on the mating behaviour of the drug fly *Sepsis cynipsea*. *Behav. Ecol. Sociobiol.*, 13: 75 – 80.
- Watras, C. J. and Haney, J. F. 1980. Oscillations in the reproductive condition of *Diaptomus leptopus* (Copepods: Calanoida) and their relation to rates of egg-clutch production. *Oecologia*, 45: 94 – 103.
- Wheatly, M. G. and Gannon, A. T. 1995. Ion Regulation in Crayfish: Freshwater Adaptations and the Problem of Molting. *Amer. Zool.*, 35: 49 – 59.
- Wheatly, M. G., Gao, Y and Nade, M. 2004. Integrative aspects of renal epithelial calcium transport in crayfish: temporal and spatial regulation of PMCA. *International Congress Series*, 1275: 96 – 103.
- Wickins, J. F. 1976. Prawn biology and culture. *Oceanography and Marine Biology*, 14: 435 – 507.
- Wickins, J. F. 1982. Opportunities for farming crustaceans in western temperate regions. In: *Recent advances in aquaculture*, p. 87-177. J. F. Muir, R. J. Roberts, (Eds.) Croom Helm, London.
- Wigginton, A. J. 2005. Some Effects of Cadmium on Select Crayfish in the Family Cambaridae. PhD. Dissertation, University of Kentucky, USA.
- Wilkins, L. A., Schmitz, B. and Herrnkind, W. F. 1996. Antennal Responses to Hydrodynamic and Tactile Stimuli in the Spiny Lobster *Panulirus argus*. *Biol. Bull.*, 191: 187 – 198.
- Willis, S. A. and Berrigan, M. E. 1977. Effects of stocking size and density on growth and survival of *Macrobrachium rosenbergii* (de Man) in ponds. *Journal of the World Mariculture Society*, 8: 251 – 264.
- Wilson, E. O. 1975. *Sociobiology*, Belknap Press of Harvard Univ., Cambridge, MA, pp. 176 – 241.
- Wong, F. and Brock, J. 1991. Takuji Fujimura 1924 -1990. *World Aquaculture*, 22 (2):75.

- Wyatt, T. D. 2003. Pheromones and animal behaviour: communication by smell and taste. Cambridge, Cambridge University Press.
- Xiaoyun, L., Wei, X. and Zhenmin, B. 2003. Histology and Functions Study of the Antennal Gland of *Penaeus chinensis*. Journal of Ocean University of Qingdao, 33 (6): 854 – 860.
- Yano, I., 1985. Induced ovarian maturation and spawning in greasyback shrimp, *Metapenaeus ensis*, by progesterone. Aquaculture, 47: 223 – 229.
- Yano, I., 1987. Effects of 17 α -hydroxy-progesterone on vitellogenin secretion in Kurma prawn, *Penaeus japonicus*. Aquaculture, 61: 49 – 57.
- Zeil, J. and Hemmi, J. 2006. The visual ecology of fiddler crabs. J. Comp. Physiol., A. 192: 1 – 25.
- Zimmer-Faust, R. K. 2000. Importance of Chemical Communication in Ecology. Biol. Bull., 198: 167.
- Zimmer-Faust, R. K. and Butman, C. A. 2000. Chemical Signalling Processes in the Marine Environment. Biol. Bull., 198: 168 – 187.
- Zimmermann, S. and New, M. B. 2000. Grow-out systems-polyculture and integrated culture. In: Freshwater Prawn Culture: The Farming of *Macrobrachium rosenbergii* (ed. By New, M. B. and Valenti, W. C.), pp. 187 – 202. Blackwell Science, Oxford, UK.
- Zulandt Schneider, R. A., Huber, R. and Moore, P. A. 2001. Individual and Status Recognition in the Crayfish, *Orconectes rusticus*: The Effects of Urine Release on Fight Dynamics. Behaviour, 138: 137 – 153.
- Zulandt Schneider, R. A. and Moore, P. A. 2000. Urine as source of conspecifics disturbance signals in the crayfish *Procambarus clarkii*. J. Exp. Biol., 203: 765 – 771.