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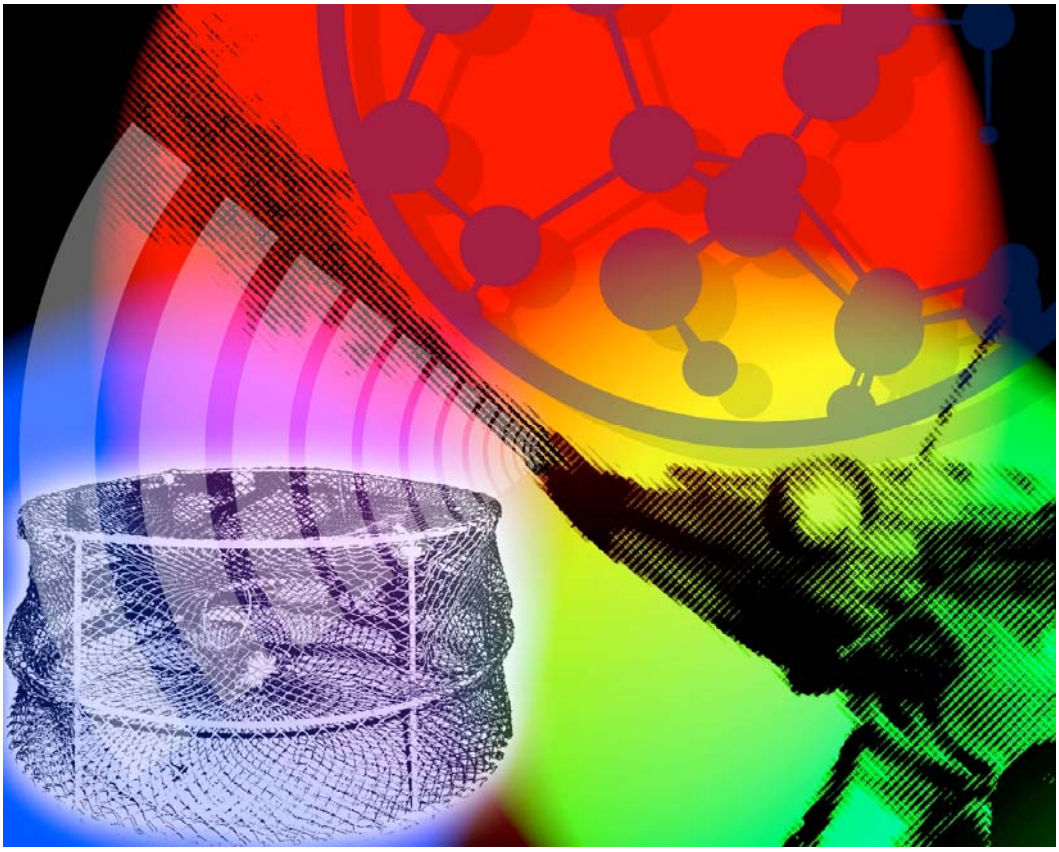

The Development of Manufactured Attractants as a Means to Harvest Prawns Specifically

Mike Hall, Neil Young and Matt Kenway

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Cover designed by Tim Simmonds (AIMS Science Communications)

CONTENTS

LIST OF FIGURES

LIST OF TABLES

NON-TECHNICAL SUMMARY

2000/256	The development of manufactured attractants as a means to harvest prawns specifically

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OBJECTIVES:

1. To quantify the attraction and specificity of pheromone compounds in experimental environments.
2. To develop methods suitable for extracting and isolating pheromones from prawns.
3. To identify a mechanism for manufacturing a bait incorporating these novel attractants.

OUTCOMES ACHIEVED TO DATE

The development of an alternative non-trawl harvesting method for penaeid prawns potentially includes a pot-trap fishery. Food baited pot-traps are used commercially to harvest several prawn/shrimp species in the north Pacific and north Atlantic. The primary bait used in pot-traps includes some sort of chemo-attractant from food. However, in some cases, including a species of crab and lobster, a type of pheromone baited pot-trap is used. Pheromone baited traps are also used in the terrestrial environment as a form of biological control with minimal environmental impact sustainable technology for capturing insect pests, which is being increasingly applied. This study examined evidence that penaeid prawns use pheromones and whether these could be isolated and identified to develop a pheromone baited pot-trap harvest technology.

The project demonstrated that penaeid prawns have a well-developed sense of smell and that a significant sexual dimorphism exists in adult animals. Males have a sense of smell several times greater than females but this difference only develops at the time of sexual maturity. Males also have larger areas of the brain devoted to processing olfactory information. Penaeid prawns have rosette glands that are believed to be the origin of reproductive pheromones. The well-developed olfactory system of sexually mature penaeid prawns lends significant support to the probability that pheromones are used to attract a distance mate.

A purification strategy for pheromones ideally requires a bioassay in order to follow a particular chemical pheromone candidate to isolate it. However, penaeid prawns lack a definitive stereo-typed courtship or any significant reproductive behavioral response. Despite several approaches to overcome this challenge it was not possible to identify the presence of a reproductive pheromone in a water sample at any specific time. However, chemical analysis of urine samples from prawns, believed to be the main carrier of far-field pheromones, revealed that females produce over 20 sex specific chemical compounds that may act as reproductive pheromones. As insufficient quantities of these potential pheromone candidates could only be accumulated they could not be fully characterised so their molecular nature remains unknown.

Overall, the data clearly demonstrates that there is a significant sexual dimorphism in olfactory ability and the production of sex specific molecules in penaeid prawns. The evidence supports the likelihood that males are attracted to a female conspecific expressing a reproductive pheromone. However, as no pheromone standard could be produced it was not possible to examine their ability to act as an attractant or to demonstrate whether or not penaeid prawns would locate a point source of purified pheromone and enter a pot-trap baited with such a compound.

KEYWORDS: Pheromone, penaeid prawn, olfaction, pot trap, alternative harvesting.

ACKNOWLEDGEMENTS

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Thanks to David and Renate Sandeman for assistance with neurophysiological recording and electron microscopy and histology preparations at both AIMS and the University of New South Wales (UNSW). Thanks also to Cedric Robillot and Rick Willis for assistance with mass spectrometry analysis, to David Sheumack with purification strategy development, to Liz Howlett for final manuscript preparation and an anonymous reviewer. Finally, a special thanks to the AIMS Tropical Aquaculture Facility staff that assisted with maintaining prawns and designing experimental systems.

BACKGROUND

Introduction

The wild prawn harvest in Australia has an average annual value of $412 million, or 17%, of total fisheries production (ABARE 2004). A significant proportion of this originates from Queensland, representing an average of 35.6% of the total Australian wild catch of approximately 25,161 tonnes landed annually. All of the Australian wild prawn commercial catch is harvested by ground trawling. In recent years particular attention by some groups focussed on the appropriateness of ground trawling in the World Heritage listed Great Barrier Reef Marine Park (GBRMP). The final report on the "Environmental Effects of Prawn Trawling in the Far Northern Section of the Great Barrier Reef: 1991-1996" indicated that trawling results in by-catches of between 37 to 55 kg per hour of trawling, that 60 to 72% by weight of this by-catch was fish, most under 30 cm in length, with the rest being invertebrates. Further, this report found that 80% of the by-catch is killed. A more recent report on the effects of trawling in Australia, concluded "The Queensland Fishery Management Authority (QFMA) proposed plan (for the Queensland Trawl Fishery East Coast and Moreton Bay) proposes to reduce maximum effort in the fishery to sustainable levels.... However, the sustainable levels referred to are for prawn stocks, not the environment, seabed habitat or by-catch. We recommend that the sustainability of the seabed habitat also be taken into account" (Poiner et al.1999). Partially in response to the concerns of the specificity of trawling and its impact on benthic marine biodiversity, significant changes have been implemented by the trawling sector. Marine macrofauna by-catch has been eliminated due to the development and deployment of turtle exclusion devices (TEDs). Further developments in by-catch reduction devices (BRDs) may result in proportional decreases in by-catch, with estimates of up to 20% (Northern Prawn Fishery Management Advisory Committee 2003). A more recent method to further reduce by-catch removal from the ecosystem involves the use of sorting bins on hoppers fitted with flow-through seawater. By sorting the trawl catch within seawater much of the by-catch can be returned to the sea alive in the vicinity of where it was captured. Devices and technologies such as these, and other proposed changes, such as the reduction of ground chain diameters, may significantly reduce the variable impact of trawling on the benthic ecosystem.

Although the environmental impacts of fishing take place against a background of natural disturbance, the impact of ground trawling on non-target benthic species continues to raise concern elsewhere around the world (Jennings and Kaiser 1998). It has been reported that the most dramatic ecosystem impacts of fishing occur on equatorial reefs and a few other specific marine environments (Jennings and Kaiser, 1998). Several countries limit ground trawling, either due to concerns of its' impact

on traditional fisheries or potential adverse impacts on benthic ecosystem food webs, especially in sensitive marine areas (see web sites on trawling bans in references). For example, Indonesia banned trawling around Java, and in 1983, in the entire sea surrounding the Indonesian region with the exception of Irian Jaya (http://www.american.edu/TED/jpshrimp.htm). Bans on inshore trawling in specific local areas have also been implemented in countries such as the USA, India, Australia and parts of Europe
(see http://www.pbs.port.ac.uk/econ/cemare/trawlban.htm
http://gurukul.ucc.american.edu/ted/JPSHRIMP.htm
http://www.marsource.maris.int/research/stupro97.htm).
In these and other countries, proposals are being put forward to further extend restrictions on ground trawling activities; in a similar way that drift net fishing was restricted or banned.

Overall, environmental concerns and fishery regulations have had a significant impact on ground trawling fisheries and it remains a possibility that yield may decrease over the coming years. Despite an Australian catch of trawled prawns averaging 25,161 tonnes, prawns represent one of the largest single fishery import products to meet domestic demand. Each year an average of $172 million are imported into the country, exceeding exports (11,194 tonnes valued at $254 million). In most recent years imports have been increasing by 12% per annum. Some of these imports are sourced from countries believed to be using unsustainable practices and may be in breach of United Nations Convention on the Law of the Sea legislation. As the Australian wild catch of trawled prawns is unlikely to increase the only alternatives to increased domestic yield and production is either through aquaculture or alternative harvesting technologies.

Under the United Nations Convention on the Law of the Sea (1994) legislation, of which Australia is a signatory, there are legal obligations which impact on fisheries management. Signatory nations are obliged to "protect and preserve the marine environment" (Articles 192 and 193, Part XII Protection and Preservation of the Marine Environment) as well as "to ensure through proper conservation and management measures that the maintenance of living resources in the exclusive economic zone is not endangered by over-exploitation" (Articles 61 and 62, Part V Exclusive Economic Zone). Governments are also obliged "in taking measures to prevent, reduce and control pollution of the marine environment resulting from the use of technologies under their jurisdiction or control.....which may cause significant and harmful changes...." (Article 196). The Commonwealth's Ocean Policy and the planned amendments to Schedule 4 of the Wildlife Conservation Act (1995 - export controls) provide further evidence that fisheries and environmental agencies will be increasing the level of regulation and compliance of fisheries. This will come at an increased regulatory financial cost to industry. A likely scenario is that as costs

increase only the most profitable, largest and most vertically integrated operators will remain. If community and cultural values inherent in today's fishing industry are to be maintained then new methods that allow ecologically sustainable development (ESD) and lower harvesting costs need to be developed to maintain the participation of the owner-operator from small coastal communities.

Current Fisheries Practices

There are few alternatives to wild fisheries harvest of prawns by means other than trawling. Some form of mid-water or off-bottom trawl gear might be suitable to harvest species of prawns that form large aggregations, or schools. For example, in Queensland, banana prawns, *Fenneropenaeus merguiensis* (systematic genera names for prawns that of Farfante and Kensley 1997), with an average value of $6 million/annum, form such aggregrations. However, the most valuable prawn sector includes tigers (*Penaeus esculentus, P. semisulcatus*), endeavour (*Metapenaeus endeavouri / M. ensis*), and king prawns (*Melicertus plebejus, M. longistylis, M. latisulcatus*) (average value of $70 million/annum - over 10 times that for banana prawns). These species do not aggregate to the same degree as banana prawns and presently the only practical means of harvesting these primarily benthic species is by ground trawling. Any development of an alternative harvesting methods for prawns is going to require a radical lateral approach (Kristjonsson 1969).

The Food and Agriculture Organisation (FAO) lists 342 species of prawns that are of commercial importance. Under half of these (155 species) are within the Infraorder Penaeidea, predominantly tropical and sub-tropical penaeid prawns, with the rest (187 species) being within the Caridea, mainly temperate to cold water pandalid prawns. Commercial scale pot trap fisheries exist for pandalid prawns in the north Pacific off British Columbia, Canada and Alaska, USA, and in the north Atlantic off Nova Scotia, Canada, and Maine, USA (Barr and McBride 1967). Of the 7 commercial species in the north Pacific, 6 of them enter pots but only 3 are extensively harvested by pots (Fisheries and Oceans Canada 1999). Some species inhabit areas where the topography prohibits ground trawling. However, ground trawling continues to be the most effective harvest technology for those species that are small and inhabit sandy and muddy bottoms (Kristjonsson 1969, Low 1999). The spot prawn (Pandalus platyceros) pot trap fishery in Canada has approximately 260 licences, with an average of 20,000 trap hauls/vessel/year harvesting 1200 tonnes with a annual value of $35 million. In contrast, a pot fishery for tropical penaeid prawns has not been developed to any significant degree (Yates 1968).

In north Queensland the commercial trawl industry targets 7 of the 22 species found in the Great Barrier Reef Marine Park. Although the entire industry is based on trawl harvesting, some species are known to enter baited pots (Racek 1955, 1959). The

economics of a trawl fishery compared to a trap fishery have been analyzed in Australia and overseas and generally demonstrate that pot trap fisheries are economically viable (see Buckworth 1993, 1995 and Morrison et al.1998). Of the variables determining economic feasibility, catch rate (kg) per pot is paramount. Major pot trap fisheries for crustacea already exist in Australia. The rock lobster pot trap fishery of western Australia is the single most valuable fishery in the country. Significant pot harvests of rock lobsters also occur in South Australia, Tasmania, Victoria and New South Wales while in northern Australia there is a large recreational and commercial catch of mud crabs from pots. Although there is no prawn trap fishery in Australia the pandalid prawn fisheries of the north Pacific and Atlantic are economically viable. A limited pot fishery for deep-water penaeid prawns also exists for some Pacific island nations (King 1981). The development of a pot trap fishery was attempted in Indonesia with limited success (Barus 1989). In Australia, the feasibility of a pot trap fishery for penaeid prawns was also examined in the Gulf of Carpentaria in the 1990's but met with no success, although part of this may have been due to it limited scope, scale and timing of trials (Buckworth 1993, 1995). In contrast, pot traps are used to harvest Kuruma prawns, *Penaeus japonicus*, from aquaculture ponds. However, a prawn pond has extremely high densities of prawns compared to the wild. In addition, as the prawns in a pond have been fed for many weeks, they may be trained to readily aggregate at the point where feed is added to the pond. Nevertheless, at least one species of penaeid prawn readily enter pots in specific conditions.

All of the pot trap fisheries for prawns, crabs, crayfish and lobsters, have been developed using food as the attractant. Pandalid and penaeid prawns have similar foraging habits so, from a biological viewpoint, penaeids prawns should also be attracted by food bait. However, where food baited pots were trailed on penaeids few prawns were captured (Racek 1955, 1959, Buckworth 1993, 1995). In addition, in the largest known trial in Indonesia there was a significant by-catch of non-target species, especially fish (Barus 1989). Of ecological significance was that the majority of this by-catch could be returned to the wild alive. Even though other tropical Crustacea can be captured in food baited pots, eg. mud crabs, it seems that a pot fishery for penaeid prawns based on food bait is not presently feasible (Vendeville 1990).

Alternative Harvest Technology

The adult and reproductive natural history of most decapods can be broadly characterized. Typically the sexes live separately and only pair briefly as adults, they are nocturnally active, usually solitary and often sparsely distributed in their geographical range. Pairs are formed after some stereotypic behavioural courtship display and remain together for short periods, ranging from a few minutes to weeks,

during a period of mating. After mating, the sexes usually separate with females displaying varying degrees of parental investment from simply spawning to complete paternal care.

The present ground trawl fishery targets high value adult penaeid prawns, often during periods of reproductive activity for each species. In Australian waters, 80% of female *P. esculentus* and *P. semisulcatus* prawns are inseminated (Crocos 1987a,b). All commercially harvested penaeid prawns have a closed thylecum and females must mate within hours after moulting (Farfante and Kensley 1997). In contrast, *Litopenaeus* species have an open thylecum, and mate immediately before spawning. Adult prawns moult frequently, usually every 10-15 days, except in winter when the intermoult period lengthens (Dall et al. 1990). For reproduction to be successful in closed thleycum prawns mating must occur at moult that takes place during hours of darkness and often in very turbid waters. Since, in the wild, the population density of many species of penaeid prawns is low, the mechanism by which males and females locate each other in order to mate must be extremely efficient. Vocalization is not likely as prawns cannot 'hear' underwater, although they can detect vibrations (Strickler 1998). There is a considerable body of evidence indicating that crustacea locate each other by chemoreception with the release of a chemical signal, exocrine or pheromone (Greek for ‘carrier of excitement’), from the sender and the reception of this chemical via chemosensors by the receiver. It is believed that pheromones play a significant role in the searching for mates and are responsible for their ability to efficiently locate each other.

Chemical Signalling in the Marine Environment

Chemical molecules can serve as external signals in the marine environment and play a profound role in its biodiversity and ecology. Chemical signalling in the marine environment has a dominant role in inter- and intra-species physiology and behaviour from single cell to multicellular organisms (Carr 1988). Specific chemicals in the environment can determine community structure and are of critical importance in the behavioural interactions of individuals in their specific environment. Chemical signalling has a major role in the life hisotry of marine organisms, such as feeding, including attractants and deterrents, avoiding predators, social interactions with other species and conspecifics, resource procurement, reproducing and migrating, fertilization, larval settlement and metamorphosis as well as long distance migration (Carr 1988). Chemical signals in the marine environment have probably evolved from parallel roles that they play in the ‘internal aquatic environment’ as cell to cell signalling molecules and neuroactive agents in multicellular organisms. Many symbiotic associations, including those between corals and zooanthellae are maintained in part through chemical communication (Paul 1992). Despite the overwhelming importance of chemical communication in the marine environment it

is one of the least understood processes ranging from large scale, such as community structure and resultant biodiversity, to the molecular scale, with chemoreception being the least understood of the major senses (Atema et al. 1988, Hay 1996).

Chemical signals play an important role in the life of many crustaceans (Atema and Voigt 1995). Whereas chemical signals from bait attracts organisms for feeding, pheromones from con-specifics attract organisms for behavioural and reproductive reasons (Breithaupt and Atema 1993, Crisp and Meadows 1963, Karavanich and Atema 1998). Examples of waterborne pheromones exist from various crustacean taxa (Bushmann and Atema 1993, Bushmann and Atema 1996, Kamiguchi 1972, Ingvarsdottir et al. 2002). Dating back as far as 1940, Hudinaga (1942) was asking questions about the ability of *P. japonicus* to find one another in the open ocean. He collected freshly moulted females (>1000) to examine them for signs of mating. Less than ten of those examined had not mated, indicating that even the prawns must have an exceptional method of locating a partner at times of mating. In the penaeids we see a high success in mating even though there is only a small window of opportunity. Since mating in most penaeid prawns occurs immediately post-moult, and the vast majority of females in the wild are inseminated, it is envisaged that the pheromone is highly potent and will be of a highly soluble, and perhaps volatile, nature. The pheromone should only attract adults but may attract both sexes (Rittschof 1990). The most extensively studied and understood reproductive pheromone systems are those of insects, especially moths (Ayasse et al. 2001). Some insect pheromones can attract conspecifics from significant distances (Vickers and Baker 1994). The powerful attractiveness of insect pheromones has led to the development of pheromone-baited traps as a means of sustainable pest control using chemical ecology (Pickett et al. 1997, Howse et al. 1998). Pheromone traps are also beginning to be investigated for marine crustacea (Luntz 2001, Hay 2002). As insects are close relatives to crustacea, which utilise pheromones, it may be feasible to develop pheromone baited traps by which to capture prawns.

History of Application Development

Harvest technologies for prawns are presently limited to trawling, which accounts for the vast majority of harvest together with a smaller, but significant harvest now coming from pot trap fisheries that use food as bait. Due to potential additional and future restrictions on prawn trawling the principal investigator was approached by environmental groups who wished to explore the possibility of developing an alternative means of harvesting prawns with minimal impact on the marine environment. After several meetings the consensus view was to develop a project to investigate the pot trap fishery based in the northern hemisphere but with a view to examining the use of pheromones as bait instead of food. The reason for the targeting of pheromones was due to findings from preliminary work in Australia (see

references of Buckworth) that indicated that in the wild penaeid prawns are not efficiently attracted to pots using food bait. Although exploratory the view of the environmental groups was that Australia must use its technological expertise and invest in this type of research given the potential ecological benefits if successful. The trawl industry view was that the development of a new low-impact prawn harvesting technology would be clearly desirable and that the proposal was generally met with a positive response. It was requested that existing operators should have the first opportunity to be early adopters of this technology if it proved successful.

Proposal Development Path

In view of the high level of technology required to successfully examine whether pheromones could be used as bait for pot traps a phased approach was adopted. This proposal, as stage one, set out:

1) to offer an initial demonstration that it is possible to attract prawns to a point source by a pheromone;
2) extract, isolate and characterize the pheromone;
3) demonstrate that prawns will enter a pot baited with pheromone.

If stage one was successful, a second phase, as another application, and in direct association with regulatory agencies (Great Barrier Reef Marine Park Authority (GBRMPA) and prawn fishers (QCFO Trawl Committee), would undertake pilot trials of the use of the pheromone bait in pot traps in coastal waters. In the first instance these trials would utilise the pot designs used in the north Pacific and Atlantic prawn pot industry. These trials would examine catch rates in various habitats and topographies in association with GBRMPA and QCFO.

The final phase would concentrate on the commercialisation and acceptance of this technology to either supplement or displace trawling in specified areas as deemed appropriate. In the north Pacific and Atlantic both mobile (trawling) and fixed (pot traps) gear operations occur in the same regions under multi-use fisheries plans. In these regions the harvest method is dependent on a number of considerations, such as topography, catch efficiency, marine ecosystem issues, etc. Prawns harvested by pot traps obtain a significantly higher value since they are landed in a pristine state. This has resulted in an overseas luxury niche market for pot trapped north Pacific and Atlantic prawns.

This application takes a strategic approach in seeking an alternative for the ESD of prawn harvesting which is specifically developed to only capture the targeted species with minimal impacts on the ecosystem and its foods webs. It offers the promise of tools that will allow future flexibility in meeting current and future

environmental challenges to meet harder ESD criteria as set down in national and international law.

The proposed development of a prawn pot trap industry based on a pheromone bait has the potential to revolutionise this fishery harvest sector. Due to its as yet unproven status the original application had a lifetime limited to 2 years. The 2-year milestone offered sufficient time and data collection to determine whether the approach is worthy of further support and continued development. If successful, later research priorities for future projects would include the identification of mass production of pheromone baits and the design of pots specifically for penaeid prawns.

NEED

This proposal represents an attempt to develop alternative technologies to harvest penaeid prawns. The proposal is not without risk, but reflects a genuine need to consider alternative fishing approaches outside the current thought envelope. If successful it would allow the industry to claim high environmental standards, meeting or exceeding the community expectations.

Exploration of alternatives to established fishery harvest methods are desirable to meet ecologically sustainable development (ESD) requirements as dictated by international (UN) and national legislation (both federal and state) covering the marine environment. For example, in recent years drift nets have been banned in many areas due to their detrimental impacts on non-targeted species and ecosystem structure. Similarly, ground trawling has been identified as a harvest technology that requires either restriction or banning due to its putative detrimental impacts on benthic ecosystems and disruption to food webs. Whereas drift net has been largely replaced by the sustainable and targeted method of hook and line harvesting, there are few alternatives to ground trawling.

Traps are used extensively for the harvesting of crustacea. Suitably designed pot traps can result in a minimum of by-catch and target individuals of specific size classes. However, pot trap fisheries utilise food as bait by which to attract the targeted species into the trap. Baited traps and pots are used for the commercial harvesting of pandalid prawns in the east Pacific and North Atlantic, where average catch/vessel/day of fishing effort is 80-110 kg. A smaller pot fishery exists for penaeid prawns in various parts of the Pacific basin and the Caribbean. However, attempts to date to develop a large pot trap fishery based on food bait for penaeid prawns have been largely unsuccessful.

It is proposed that chemical attractants, and not food bait, be examined as a means to harvest penaeid prawns in pots. The development of alternative harvest methods could form a non-trawl fishery with minimum by-catch, open up new areas to harvesting which are unsuitable for trawling, and produce a less stressful method to collect broodstock *P. monodon* prawns for the aquaculture sector as well as have spin-off potential for the development of pot trap fisheries for other species of crustacea.

OBJECTIVE 1
To quantify the attraction and specificity of pheromone compounds in experimental environments

Introduction

Chemical signals in the marine environment can generally be classified into four categories. Primary metabolites, defined as substances used in basic metabolic processes of an organism, and as such typically as feeding attractants (Carr and Derby 1986b). For example, adenosine 5'-triphosphate (ATP) is an important intermediary of energy metabolism, and hence common to all cells, but it rapidly decays to adenosine 5'-monophosphate (AMP) as cells die. It has been demonstrated that ATP stimulates carnivory in the spiny lobster, *Panulirus argus*, whereas AMP inhibits feeding as it is assumed to indicate putrifying food (Zimmer-Faust 1993). Other primary metabolites, especially amino acids and biogenic amines are powerful feeding attractants in lobsters, crabs and prawns (Carr and Derby 1986b, Coman et al. 1996, Daniel and Bayer 1987, Daniel and Bayer 1989, Mackie 1973, Zimmer-Faust 1989). Amino acid derivatives, such as N-acetyl-D-glucosamine, are part of a patented feeding composition acting as a feeding excitant, attractant and incitant for crustacea, including penaeid prawns (Cardinale et al. 1999). Secondary metabolites, defined as chemicals constructed by the condensation of primary metabolites into more complex structures, can act as chemical signals that can be unique and hence be more species specific and unique (Trapido-Rosenthal 2001). These chemical signals can be detected by an organism on the two-dimensional space of a solid surface, such as larval settlement cues (Table 1.1) and feeding deterrents, which are common to sessile 'defenseless' soft-bodied benthic organisms (Daloze et al. 1980, Wolfe et al. 1997, Zimmer-Faust and Tamburri 1994). Secondary metabolite based feeding deterrents include terpenes, alkaloids, halogenated compounds, polyphenols, polyketide and steroid glycosides and prostaglandins (Carr 1988). Other chemical signals that may act in a two-dimensional space, include those which influence habitat selection by pre-settlement larvae (Crisp 1974, Morse 1984). However, both feeding deterrent and larval settlement chemical signals may also be released into the three-dimensional space of the water column and hence can act at significant distances from the original source (Atema and Stenzler 1977, Faulkner and Ghiselin 1983, Mackie et al. 1977, Kraus-Epley and Moore 2002).

Pheromones are generally defined as any chemical signals released by an organism that influence the physiology and/or behaviour of other members of the same species (Karlson and Luscher 1959, Dunham 1978, Stacey et al. 1994). Behaviour responses include alert and alarm response, aggregation, agonistic behaviour,

searching, feeding, aggression and reproductive activity. Pheromones have been classified according to their presumed biological function (Table 1.1). Reproductive pheromones may be subdivided into two categories. The first includes pheromones that act on single cells from multi-cellular organisms, for example gametes, and includes sperm attractants, of which the best studied are from algae (Boland 1995). The second are those that operate on sexually mature adults associated with reproduction, for example those that induce behaviours such as searching for a mate, courtship and mating and maternal behaviour (Dunham 1988, Lass and Spaak 2003).

Table 1.1. Examples of pheromones from the marine environment: their function, estimated operational distance and chemistry.

FUNCTION	OPERATING DISTANCE (IN METRES)	MARINE ORGANISM	CHEMICAL IDENTITY	REFERENCE
Feeding				
Food location	$10^{0} – 10^{2}$	many marine invertebrates	various – amino acids, biogenic amines	Lenhoff and Lindstedt, 1974 Grant and Mackie, 1974
Alarm response	$10^{-2} – 10^{-1}$	molluscs	navenones	Sleeper et al., 1980
Deterrent	$10^{-3} – 10^{-1}$	echinoderms	saponins	Trapido-Rosenthal, 2001
Larval				
Release	$10^{-3} – 10^{-1}$	crabs	peptide	Forward, et al., 1987
Settlement	$10^{-3} – 10^{1}$	many marine invertebrates	various, low MW, peptides	Pawlik, 1992 Hadfield and Paul, 2001
Metamorphosis	$10^{-6} – 10^{-4}$	molluscs	unknown, low MW	Burke, 1984
Reproduction				
Courtship	$10^{-1} – 10^{2}$	fish	steroid	Sorensen and Caprio, 1998
Mate location	$10^{-1} – 10^{3}$	crustaceans	unknown	Ryan, 1966
Copulation	$10^{-1} – 10^{0}$	crabs ciliates	unknown, low MW peptide	Kamio et al., 2002 Raffioni et al., 1992
Gamete release	$10^{-6} – 10^{-4}$	polychaete mollusc	peptide peptide	Zeeck et al.,1996, 1998, Zatylny et al., 2000
Gamete attractant	$10^{-6} – 10^{-4}$	algae	ectocarpene	Muller et al. 1990
Social				
Aggregation	$10^{-3} – 10^{0}$	molluscs barnacles lobsters	peptide unknown, low MW unknown	Painter et al., 1998, Crisp and Meadows, 1962, Hunt et al. 1985
Aggression	$10^{-1} – 10^{0}$	lobsters	unknown, low MW	Zulandt-Schneider et al. 1999

**estimates based on biological/behavioral response of cell/organism to putative pheromone.*

It has been over a century since behavioural studies established that crustaceans can detect chemical stimuli in the environment and that chemoreceptors are concentrated on specific appendages (Nagel 1894). It wasn't until the 1960s that studies revealed the basic organisational structure of crustacean chemosensory systems from the level of the receptor cells themselves to the neural receptors organs (Laverack 1963). Major reviews of crustacean chemoreception include that of Barber (1961) and Ache (1982). Since that time only specific areas of crustacean chemoreception have been reviewed, for example Atema et al. (1988), Hay (2002) and Stacey et al. (2002). In order to realistically appraise the feasibility of developing pheromone baits for pot traps it is critical to understand and review the available evidence of chemoreception and pheromones in Crustacea.

REPRODUCTIVE PHEROMONES IN CRUSTACEA

Historically, some of the strongest evidence for reproductive pheromones in marine organisms has been obtained with Crustacea (Mackie and Grant 1974, Dunham 1988, Snell and Morris 1993, Ingvarsdottir et al. 2002). In this phylum the most widely studied pheromones are the reproductive pheromones within the subclass Copepoda and Malacostraca, and specifically the order Decapoda, including lobsters (suborder Macrura reptantia) and crabs (infraorder Brachyura). In the common prawn (*Palaemon serratus*), the northern shrimp (*Pandalus borealis*) and the shore crab (*Carcinus maenas*), evidence for a contact pheromone has been documented (Forster 1951, Carlisle, 1959, Edwards 1966). Pheromones, typically originating from the female, but not always, have been implicated in mate searching and reproduction in many of these (Dunham 1978, 1988). In general, the most convincing evidence for reproductive pheromones is in solitary species that mate immediately after the female moults, as is the case in closed-thylecum penaeid prawns. There is also evidence for both far-field mate searching pheromones and near-field contact, courtship, and mating pheromones.

CLASS MAXILLOPODA, SUBCLASS COPEPODA

Evidence for reproductive pheromones in relation to mate location has been reported across the Copepoda, including calanoids (Katona 1973, Griffiths and Frost 1976), cyclopoids (Uchima and Murano 1988), harpacticoids (Lazzaretto et al. 1990), and a siphonostomatoid (Richie et al. 1996). As found in other Crustacea, these studies have demonstrated that mate attraction can occur from a distance and that recognition of the reproductive status of the female occurs after tactile chemoreception. Although not chemically characterized it is believed that some of these pheromones are species- and sex-specific (Kelly et al.1998).

CLASS MALACOSTRACA, ORDER DECAPODA, INFRAORDER ASTACIDEA AND PALINURA

Evidence of reproductive pheromones comes from studies on the American lobster *Homarus americanus*, (McLeese 1970, Atema 1986), Scalloped spiny lobster, *Panulirus homarus* (Berry 1970), Cape rock lobster, *Jasus lalandii* (Rudd and Warren 1976) and the freshwater crayfishes, *Procambarus clarkii, Orconectes virilis, O. propinquus and Astacus leptodactylus* (Ameyaw-Akumfi and Hazlett 1975, Tierney and Dunham 1982, Breithaupt and Eger 2002). Unlike many other Crustacea, it is the female lobster that actively searches for a mate, rather than the male searching for the female, and is based on *in situ* observations of lobsters in their natural habitat (Atema 1986). To attract females the male lobster fans a strong current with his pleopods through his shelter and into the environment together with a putative pheromone to attract females. Females orientate towards burrows in response to pheromones emitted by the male occupant (Cowan and Atema 1990). During the next phase of the mating ritual the female may also produce pheromones that she releases in urine, directed towards the male. The female may also produce a 'contact' pheromone, which requires physical contact, which can modify male behaviour. If the male determines that the female is approaching a moult, and is reproductively mature, the male allows the female into his burrow where she remains for one to two weeks, protected from predation. Copulation usually occurs within 30 minutes of the female moulting (Cowan and Atema 1985). Although this matinig ritual is well understood the chemical nature of these putative pheromones is unknown.

Pheromones released by the Caribbean spiny lobster (*Panulirus argus*) are attractive to conspecifics. In the commercial fishery for these lobsters traps are baited using live *P. argus* lobsters to increase capture rates (Hunt et al. 1985). There is speculation that the pheromone in this case may be associated with aggregating individuals of the same species rather than a reproductive pheromone associated with mate attraction. The hypothesis has some support in that many species of spiny lobsters are gregarious and prefer shelter where there are other conspecifics. By contrast many other crustaceans, including some crabs and penaeid prawns, generally live in isolation from each other for most of their lives. Regardless of life history, adult crustaceans must associate with each other during the breeding season for successful mating.

CLASS MALACOSTRACA, ORDER DECAPODA, INFRAORDER BRACHYURA

In brachyuran crustaceans chemical signals that elicit mate location, courtship display and precopulatory mate guarding have been reported (Bushman 1999, Dunham 1988, Kamio et al. 2002). In *Callinectes sapidus* both males and females respond to pheromones from conspecifics (Gleeson 1991). Pubertal females release pheromone(s) that evoke a specific courtship display and cradle-carry behavior in

male crabs. In tests, including control seawater, with and without urine from adult males and females containing a putative pheromone, and visual displays of adult males and females, only the female exhibits a response (Teytaud 1971). Females only respond when putative pheromone from a sexually mature male is combined with sexual displays, but not when only pheromone or displays alone, are presented (Teytaud 1971). Post-pubertal moult crabs do not appear to release pheromone(s) and do not necessarily result in a response from males (Gleeson 1980). Reproductive pheromones appear to also originate from the male and when combined with a visual display (dance) from the male, elicit a receptive response only in pre-moult females (Gleeson 1991). Studies on *C. sapidus* tentatively identified the presence of three pheromones in female urine that are attractive to males but no pheromone has been characterised (Gleeson 1982, Gleeson et al. 1984). These behaviours appear to be regulated by pheromones from both urine and non-urine sources (Bushmann 1999). It is not known whether these pheromones function in distance mate location and short distance courtship and reproductive assessment. In the crabs *Carcinus maenas* (Eales 1974, Seifert 1982), *Portunus sanguinolentus* (Christofferson 1970, Ryan 1966), *Telmessus cheiragonus* (Kamio et al. 2000), *Erimacrus isenbeckii* (Sasaki 1995) and *Cancer magister* and *Pachygrapsus crassipes* (Takahashi 1975), evidence suggests that pre-moult females produce a pheromone that elicits a chemokinetic response from males and that it is sex-specific (Ryan 1966, Eales 1974).

All the above mentioned crabs express a stereo-typed sequence of reproductive behaviours from initial mate location, initiation of precopulatory guarding, followed by moulting and copulation. Kamio and colleagues developed a 'sponge assay' in which a small aliquot of dissolved putative pheromone was absorbed onto a small cube of sponge that was then presented to males in a confined aquarium (Kamio et al. 2000). They demonstrated that there are at least two distinct pheromones released from pre- and post-moult females. The pre-moult pheromone only elicits 'grasping' behavior in the male whereas the post-moult pheromone induces copulation. They also demonstrated that at least one of the pheromones produced by female *T. cheiragonus* was released in the urine, as a sponge assay containing the surface secretions of a female did not elicit a response from males. In addition, these researchers believed the pheromone to be released in an episodic manner and may be associated with mate searching. Its chemical indentity is not known but is less than 1,000 Da (Kamio et al. 2002). Using the same sponge assay as described above, Asai and colleagues, using a bioassay directed purification strategy have isolated a number of ceramides from female *E. isenbeckii* which elicit mate guarding behaviour (Asai et al.2000, Asai et al.2001). However, as penaeid prawns do not exhibit such stereo specific behavior the 'sponge' assay, so valuable to pheromone research on crabs, is not a viable option.

In the blue swimmer crab (*Callinectes sapidus)* commercial fishery of Chesapeake Bay, USA, pots are baited with reproductively mature males to attract females (Warner 1976). Pots baited in this fashion have an increased catch rate compared to food only baited pot traps. After baiting pots with two or three males catch rates of up to twenty to thirty females per pot are reported. This is a clear example of a commercial fishery using pheromone baited traps to improve catch rates at certain times of the year. It should be noted that it is also possible to catch these crabs at other times of the year in unbaited 'bare' pots. If pots are placed in open areas lacking natural refuges, immediate pre-moult crabs will enter pots to moult as the pot offers protection when a soft-shelled crab is extremely vulnerable to predators.

CLASS MALACOSTRACA, ORDER DECAPODA, INFRAORDER CARIDEA

Several caridean shrimps have been reported to use a pheromone to mediate mating and courtship behaviour. These include the freshwater prawn, *Palaemon paucidens* (Kamiguchi 1972), the marine snapping shrimp, *Alphaeus heterochaelis* (Schein 1975), the Harlequin shrimp, *Hymenocera picta* (Seibt and Wickler 1979) and a Pacific shrimp, *Heptocarpus paludicola* (Bauer 1979). Most of these researchers concluded that, at the very least, a contact pheromone was involved in courtship and mating. More extensive studies by Kamiguchi (1972) presented evidence of a pheromone released by the female in the freshwater shrimp *P. paucidens.* In this species breeding is restricted to once a year and undergoes a special parturial moult with mating occuring within 8 hours but with loss of attractiveness within 30 minutes after the moult (Kamiguchi 1972). Chemokinetic reactions are induced in males exposed only to water from post-parturial moult females and not from non-parturial moults indicating the transient production of a putative pheromone from the female. If additional visual and tactile stimuli were present, other reproductive behaviours, such as mounting and copulation, occur.

CLASS MALACOSTRACA, SUBCLASS EUMALACOSTRACA, ORDER AMPHIPODA

The amphipods lend themselves to scientific studies on pheromones as they readily adapt to controlled laboratory conditions. As a consequence the reproductive behavior of some species has been described in detail (Borowsky 1983, Hartnoll and Smith 1980). Today they are one of the best studied groups of Crustacea with regards to their sexual biology (Dunham and Hurshman 1991). Amphipods are usually solitary and individuals only transiently come together for the purpose of mating. As with many other decapod Crustacea, breeding is seasonal and mating in gammarid amphipods must occur within a few hours (3 to 4 hours) after moulting (Borowsky 1991). In *Gammarus palustris* ovulation does not occur until 48 hours after moulting, even if copulation takes place, and hence success in reproduction is a very transient event (Borowsky 1983). However, unlike some other crustaceans, females do not store sperm and therefore must be accompanied by a male at moulting for

fertilization to be successful. Males mate guard females for a few days immediately before a pending moult and therefore seek out pre-moult females. Soon after mating males depart.

Pheromones from females can attract mates from a distance. In *G. palustris* and the tube-building amphipod *Microdeutopus gryllotalpa*, males react chemokinetically to a chemical signal from pre-moulting female conspecifics with an increase activity in mate seeking (Borowsky 1984, Borowsky 1991). In the euryhaline amphipod *Gammarus lawrencianus*, pre-moult mate searching and guarding also occurs (Dunham and Hurshman 1991). In *Gammarus duebeni*, there is sexual dimorphism in the second antennae with males processing a single row of dorso-medial transparent club-shaped appendages, which are innervated by sensory cells. These are absent in females (Dahl et al.(1970 a,b). Males exhibit chemokinetic responses to water that contains females but do not respond to water that contains males. Exposure of males to radioactively labelled females, with [3H]acetyl-gucosamine, resulted in selective labelling of the second antennae of males but not when males were exposed to radioactively labelled males (Dahl et al. 1970b). The studies by Dahl and colleagues offer some of the best evidence of pheromone production of sexually mature females and incorporation of that pheromone into sensory inputs into the second antennae of males. Overall the evidence in amphipods indicates that a pheromone stimulus, probably exclusively from the female, does trigger, from a distance, mate searching behavior and for it to be successful requires the release of this pheromone into a water current.

AGGREGATION PHEROMONES IN CRUSTACEA

Aggregation pheromones have been defined as any substance that induces behavior in conspecifics leading to an increase in their density in the vicinity of the pheromone source (Birch and Haynes 1982). Various Crustacea form dense aggregations including large pods by juvenile King crabs, *Paralithodes camtschatica* (Powell and Nickerson 1965), conical heaps of spider crabs, Maja squinado (Stevcic 1971), migratory columns of several species of spiny lobster, Panulirus spp. (Hernnkind 1969), swarms of mysid shrimps (Clutter 1969) and copepods (Ueda et al. 1983) and schooling in penaeid prawns (Rothlisberg et al. 1985). To what extent these aggregations are regulated by pheromones is unknown (Dunham 1988). However, there is good evidence that an aggregation pheromone influences the cyprid larvae of barnacles, although it may also be classified as a larval settlement pheromone (Crisp 1974). Aggregation pheromones have also been described for other Crustacea (Ingvarsdottir et al. 2002). Such pheromones could act as attractants for pheromone baited pot traps.

CHEMORECEPTION

Chemoreception is the least understood of the major senses, even though chemical detection is critical to the evolutionary success from bacteria to multicellular organisms and is arguably the most ancient of the senses (Firestein 2001, Strausfeld and Hildebrand 1999). Detection and evaluation of chemical signals, as well as the initial response to those signals, are all accomplished within the same cell (Trapido-Rosenthal 2001). Hence single cell organisms and single-celled gametes of multicellular organisms have been important model systems from which to study chemoreception physiology (Riffell et al. 2002, Van Houten 1998).

The existence of a targeted olfactory receptor system is a pre-requisite of any group of animals that employ chemical attractants to find one another (Ache 1988; Derby and Atema 1988). In higher eukaryotes, from invertebrates to humans, there is a highly conserved evolutionary convergence towards the organization of signalling pathways in olfactory systems (Hildebrand and Shepard 1997, Strausfeld and Hildebrand 1999). A primary olfactory system exists for sensing the general environment, for example, detecting food and predators and territoriality. However, many organisms have developed a second olfactory system specifically associated with reproduction and the procurement of a receptive mate. For example, the vomeronasal system in mammals is an independent and dedicated olfactory system specifically for species-species communication associated with reproductive processes (Firestein 2001). The invertebrate olfactory system has not specialized to such an extent but there is ample evidence that olfaction plays a pivotal role in the reproduction of many species (Krieger and Breer 1999).

An essential step in the investigation of chemical attractants in penaeid prawns is to establish the presence and nature of an olfactory receptor system, using the anatomical criteria already recognised in the decapods, and to determine if there are any sexual dimorphisms in the system which may provide clues as to whether only one partner or both are being attracted. The basic components of the olfactory system include the recognition and discrimination of olfactory signals by chemoreceptors on the organism. Received chemical stimuli, in turn, activate, with varying degrees of specificity and sensitivity, chemo-electrical transduction through peripheral neural pathways and processed by the central nervous system. This cascade of molecular to physiological events eventually leads to behavioural changes in the receiving organism. Once the anatomical nature of the penaeid olfactory system is known, its physiological properties, including its responses to various chemical attractants, both natural and synthetic, can be explored. In order to study the olfactory system of Crustacea, it is necessary to have a clear understanding of each of these components and pathways, as each component offers avenues of investigation towards the development of a pheromone-based harvesting technology.

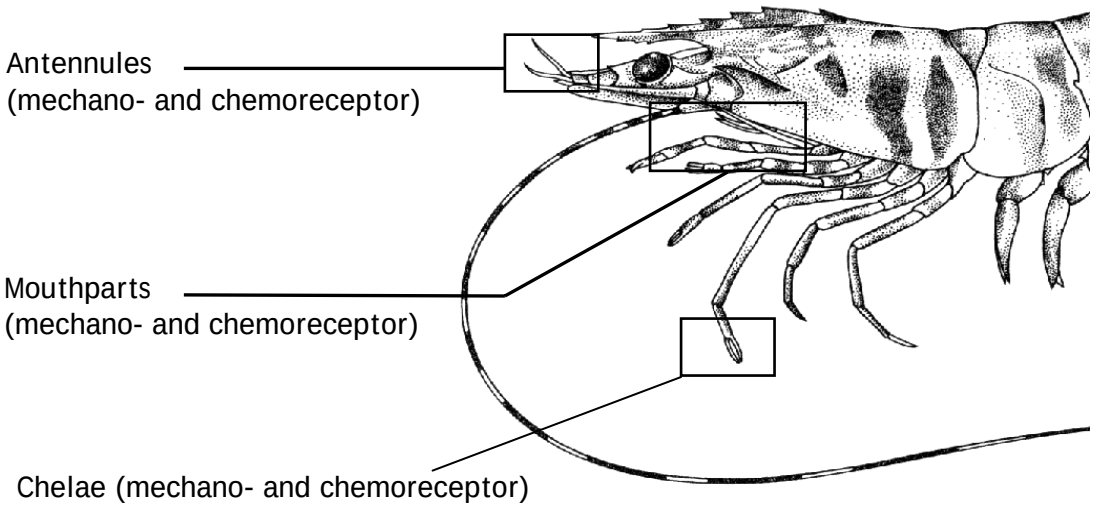


Figure 1.1. Diagram showing major chemo- and mechano-receptors sites on penaeid prawns. Although chemoreceptors are found on mouthparts and chelae the only ones associated with distance chemoreception are those found on the antennules.

RECEPTOR LOCATION

In Crustacea the external chemical environment is perceived by sensilla, most in the form of a hairlike structure on the external cuticle, that are composed of one or a few cells with a nerve connection. The primary sites for chemoreception include the lateral branch of the first pair of antennae, or antennules, the pereiopod dactyls and the mouthparts (Fig. 1.1) (Hazlett 1971). The distinct sensillium located on the antennules contain an anatomically distinctive class of sensillium called aesthetascs (Ache 1982). Some evidence exists for non-aesthetasc sensilla being chemosensory (Cate and Derby 2001, Schmidt et al. 2003). The other main class of chemosensory sensilla includes the dactyls chemo-sensillae. These are usually arranged in parallel rows or tufts along each side of the dactyl and terminate just before the epicuticular cap and may be arranged to contact objects held in the claws (Shelton and Laverack 1970). The third maxillipeds are also likely to possess chemo-sensillae (Ache 1982). Evidence for other chemo-sensillae is based on limited evidence but includes the sensory pore of the eyestalk and others (Altner and Prillinger 1980; Cate and Derby 2001). Much of the evidence for other chemosensory sensilla is largely indirect, including behavioural and physiological data, either by ablation, ultrastructure analysis or autoradiography but rarely by electrophysiological methods (Ache 1982).

In decapods the aesthetascs (antennular chemoreceptors) are the organs of smell and are readily morphologically distinguished from dactyl and mouthpart chemoreceptors, which are the organs of taste (Ghiradella et al. 1968, Ache 1982). The sensilla that come into contact with food, ie. pereiopod dactyls and the mouthparts, have a dual innervation of chemoreceptors and mechanoreceptors,

while aesthetascs have only chemoreceptors (Laverack 1988). The dactyl and mouthpart chemoreceptors mediate high-level stimulus detection immediately adjacent to, or in contact with, the stimulus that elicits consummatory behaviour. In contrast, aesthetascs mediate low-level stimulus detection at significant distances from an odor source that serves to elicit foraging or searching behaviour. Physiological evidence indicates that the antennular chemoreceptors have a stimulus threshold 4 to 5 orders of magnitude lower than those of the dactyl or mouthpart receptors (Ache 1982).

Comparative studies in Crustacea have shown that aesthetasc sensilla are distributed differently on the lateral filament of the antennules (Hallberg et al. 1997). Typically, the aesthetascs are restricted to a portion of the lateral branch of the antennules, either on the proximal part, as in mysids, or on the distal part, as in *Palinurus* species (Cronau 1978, Grunert and Ache 1988). The aesthetascs may also be spread over a large area of the antennule, as in *Crangon crangon*, in dense arrays, as in crabs and hermit crabs, or in less dense arrays as in prawns (Hallberg et al. 1997). The arrangement of aesthetascs has been correlated with antennular flicking.

The antennule 'flick' response observed in decapods is considered evidence that they are adapted for low-level stimulus detection of odors (Schmitt and Ache 1979). However, in some species antennule flicking is additionally associated with social display (Caldwell 1992). As described previously, chemoreception can be divided into two primary events from a hydrodynamic standpoint. Large-scale turbulent flow, on a scale of millimetres to many meters, determines the patchy intermittent structure of odor plumes carried from its chemical source to the antennules of the receiving organism. In contrast, small scale laminar flow near the antennule surface and molecular diffusion transport odorants to the chemoreceptors (Koehl et al. 2001). As molecular diffusion is a slow process (diffusion time increases as the square of the distance), a thick boundary layer slows down odorant access. Many Crustacea can be observed to rapidly move their olfactory antennules through the water and such behavior is termed as antennular flicking (Stacey et al. 2002). Antennular flicking results in the development of a velocity gradient in the fluid between the rapidly moving antennular surface but still with a stationary molecular (boundary) layer of fluid in contact with the antennular surface as this layer does not slip relative to the surface (Mead and Koehl 2000). The thickness of the boundary layer determines the rate of odorant transfer within thin layers, due to rapid flicking, permitting relatively rapid access to the aesthetasc surface. Fine-scale high-frequency structures of an odor plume are discrete parcels of chemical stimuli that are captured by the chemoreceptor during the rapid downstroke (Koehl et al. 2001). Lobsters can detect pulses of odorant molecules within 50 milliseconds but must be exposed to a pulse for at least 200 milliseconds in order to measure concentration, giving olfactory sampling frequency (sensitivity) of 4-5 Hz (Gomez and Atema 1996).

Such sensitivity allows a lobster to use the fine-scale pattern of odorant concentration in a turbulent flume to locate its source of origin. Antennular flicking has also been reported in the grass shrimp, *Palaemonetes vulgaris*, and is similar to that described for lobsters, but appears to alter its flicking sampling frequency as it grows (Mead 1998).

CHEMORECEPTOR STRUCTURE

Each chemosensory aesthetasc contains several hundred olfactory receptor neurons (ORNs) bundled into a sensilla. This is the functional unit of the olfactory system that have a number of common anatomical features (Hallberg et al. 1992). Aesthetascs are morphologically characterized as having a thin cuticle, lacking a terminal pore or tubules and have a central lumen containing the dendrites of chemosensory neurons filled with a lymph-like fluid (Derby et al. 1997, Gleeson et al. 1993). Each aesthetasc has a thick, laminar, non-permeable proximal region with the majority distal region containing the cilia of the olfactory neurons together with their receptors and other transduction components (Hatt and Ache 1996, Derby et al. 1997). In the spiny lobster, *P. argus*, the cuticle in the distal region is very thin, approximately 0.8 to 1 micron thick, with no obvious surface pores or tubules (Grunert and Ache 1988). However, in other species the cuticle can be as thick as 4-5 micron, such as in the crayfish, *Orconectes propinquus* (Tierney et al. 1986). In contrast to the spiny lobster, the aesthetascs of the crayfish appear to have an apical pore. However, it has been questioned whether the apical pore is an artefact of abrasion or associated with moulting (Snow 1974, Guse 1980). Whatever the case, blockage of the pores in an ostracode seta did not prevent dye penetration (Anderson 1975). In the dactyl and other sensilla of the kelp crab, *Pugettia producta*, and shore crab, *Carcinus maenas*, there are pore tubules of approximately 100-200 nm width which are large enough to allow the passage of chemicals of molecular masses of 100kDa (Hamilton et al. 1985, Schmidt 1989). Even without pores or tubules, the thin cuticle wall of the spiny lobster aesthetascs are permeable to dyes with molecular masses of 500 Da that can pass through the cuticle in under 300 milliseconds (Anderson 1975, Grunert and Ache 1988, Michel and Ache 1992). Ultrastructure and functional studies have demonstrated that the aesthetasc cuticle acts as a molecular filtering device with an upper end molecular weight cut-off of 8,500 Da with movement of molecules below this level being proportional to their molecular mass (Derby et al. 1997). Such evidence clearly indicates that any reproductive pheromone molecule would have a low molecular mass.

Many insect species have well developed reproductive pheromone sensing systems. In some Lepidoptera, the males possess enlarged antennae with numerous olfactory receptors that respond to female pheromones (Hansson 1995). In Crustacea, very few species have been reported to exhibit sexual dimorphism in aesthetascs even though

the presence of reproductive pheromones has been demonstrated (Gleeson 1982, 1991, Bamber and Naylor 1996a,b). When the antennules are ablated in male amphipods the males' response to pheromones from conspecifics females was eliminated (Ducruet 1973). From the literature published on decapod Crustacea there are only two reports of pronounced sexual dimorphism in the number of antennule aesthetasc even though a large body of work exists studying the olfactory systems (Gleeson, 1982; Hallberg *et al.*, 1997; Mead, 2002; Mead, 1998; Mead and Koehl, 2000; Sandeman and Sandeman, 1996; Steullet *et al.*, 2000). These references more commonly imply a role of sex pheromones with no observed antennular dimorphism in relation to the size of antennules and number or size of aesthetascs between the sexes. However, sexual dimorphism in aesthetascs has been reported in some galatheid decapods, amphipods and the freshwater caridean decapod, *Palaemon paucidens* (Marcus 1912, Kamiguchi 1972a,b). In species within this group, males had enlarged antennular sensory apparatus, including an increased number of aesthetascs and antenuule length. In the amphipod, *Monoporeia affinis*, adult males have at least four times as many aesthetascs as females (Hallberg et al. 1997). In addition, male-specific sensilla, usually found on the antennules but of uncertain chemosensory abilities, have been described in the Peracarida, including mysids, cumaceans and amphipods, but not in decapods (Guse 1983, Lowry 1986, Johansson and Hallberg 1992, Johansson et al. 1996). It has been suggested that the Peracarida possess two parallel olfactory systems; one a general olfactory system found in both sexes and the other a putative male-specific reproductive pheromone olfactory system (Hallberg et al. 1997). A lack of dimorphism could relate to decapod Crustacea exploiting a variety of strategies in olfactory systems, such as through plasticity in the number of aesthetascs, olfactory lobe glomeruli and olfactory receptor neurons (Beltz *et al.*, 2002). It may also relate to phylogeny, as the majority of publications regarding aesthetasc development come from species belonging to the order Reptantia, whereas *P. monodon* is within order Natantia. Further work comparing aesthetasc counts and development for both orders is required.

Olfactory receptor neurons (ORNs) are distinguished from many other sensory systems as there can be highly dynamic, regulating cellular turnover during the lifetime of an organism (Steullet et al. 2000). Unlike vertebrates and gastropods, in which the olfactory ORNs are internalised, the ORNs are indirectly discernible in Crustacea as they have compartmentalized peripheral olfactory systems in the aesthetascs. In general, changes in numbers are associated with growth but other changes may be observed (Sandeman and Sandeman 1996, Sandeman et al. 1998, Derby et al. 2003). As the aesthetascs are readily detectable and are the site of olfaction, they can offer important information on changing olfaction sensitivity of individual animals.

PHEROMONE GLAND

The site of pheromone production remains largely speculative in Crustacea. Possible sources include modified tegumental glands, so called due to their association with the integument, which exist in all species so far examined (Talbot and Demers 1993). These glands are composed of one or more specialized epidermis cells and typically form rosette glands which have a duct that opens to the exterior by an excretory pore and are believed to have an exocrine function. The general role of these glands is not well known but they are believed to at least be involved in the secretion of phenoloxidase substances assisting in the tanning of the newly formed epicuticle into a hard shell as well as producing a mucopolysaccharide like substance protecting the cuticle against abrasion and having anti-microbial properties (Copere et al. 2004). A subset of the tegumental glands, the so-called nephropore rosette glands, have been suggested to be the source of pheromones in lobsters (Bushmann and Atema 1996). The nephropore is the excretory duct of the antennal gland, which itself is the main organ of nitrogenous excretion (Talbot and Demers 1993).

In the freshwater prawn (*Palaemon paucidens*) a unique type of rosette gland, termed the sternal gland, has been described (Kamiguchi 1972). It is not associated with the antennal gland but is a diffuse gland situated on the base of the last three pairs of coxopodites of the pereiopods and ventrally beneath the thoracic sternum. It is not found in males nor juvenile females but only in adult females. It is composed of 600-800 glandular structures with ducts that tend to open to the ventral cuticle. The sternal gland contains a secretory material of unknown identity that is discharged within 30 minutes of the parturial moult. Males are attracted to females for mating only for the first 30 minutes post-moult. However, extracts of the sternal gland from mature females resulted in both males and females being attracted to it. This may either indicate that the sternal gland is not the source of a reproductive pheromone or that the pheromone must be naturally processed and released from the female to have its biological effect. Sternal glands have been reported in termites and have been shown to secrete a trail pheromone (Luscher and Muller 1960). Although not specifically examined for histologically, there is no overt sign of a sternal gland on penaeid prawns.

CHEMORECEPTION NEUROBIOLOGY

In Crustacea, aesthetascs are innervated by multiple bipolar sensory neurons with the degree of innervation varying significantly, with 300 to 500 receptor neurons per aesthetasc in *Pagurus hirsutiusculus* to only 8 to 10 per antennal seta in *Acetes* spp. (Ghiradella et al. 1968, Ball and Cowan 1974, Ache 1982). Aesthetasc neurons typically give rise to two ciliary dendrites projecting into a lumen within the setae (Guse 1978). Chemosensory neurons typically contain minuscule hair-like extensions (cilia) that project from the cell towards the receiving environment and carry the

olfactory receptors (Dennis 2004). It is believed that the two cilia per sensory cell give an increased surface area of the dendritic outer segments where the olfactory receptors are present (Hallberg et al. 1997). The dendrites branch extensively, up to an estimated branching ratio of 20 distal branches for each dendrite, resulting in 6,000 to 8,000 sensory endings filling the lumen of aesthetasc setae in *P. hirsutiusculus*, and are presumably the site of signal recognition and chemo-electrical transduction (Ghiradella et al.1968). Such extensive dendrite branching may be unique to decapod aesthetascs and results in an exquisitely sensitive olfactory system (Ache 1982).

Odorant molecules are detected by chemoreceptors located on the dendrites of olfactory receptor neurons (ORNs) (Lynn et al.1994). In insects, a large family of heterotrimetric GTP-binding protein (or G protein)-coupled receptors are involved in the recognition of odor signals (Hildbrand and Shepherd 1997, Krieger and Breer 1999). Once activated the receptors stimulate G-protein-modulated excitatory or inhibitory second messenger cascades which in turn modify membrane potential of the chemoreceptor neuron by gating ion channels either directly or indirectly through phosphorylation of proteins (Fadool et al. 1995, Gentilcore and Derby 1998, Hatt and Ache 1994). As single ORN may posses more than one type of receptor protein, second messenger cascade and ion channels, both excitatory and inhibitory, the net electrophysiology response of an ORN to an odorant chemical is complex (Ache and Zhainazarov 1995, Boekhoff et al. 1994, Cromarty and Derby 1997). In addition, once an odorant is bound to an ORN the compound might inhibit or facilitate the binding of other odorants to their receptor sites (Kurahashi et al. 1994, Olson and Derby 1995).

The final step in chemoreceptor activation includes the chemosensory neuron converting information about the odorant signal, such as its quality and concentration, into electric action potentials along the axon to higher processing centres, such as the olfactory lobes in the central nervous system, terminating in a physiological or behavioural response of the organism. Molecular, electrophysiological, biochemical and genetic research into phylogenetically diverse organisms indicate that the fundamental principles of chemoreception transduction are shared across phyla (Kreiger and Breer 1999). One of the most extensively studied organisms in chemo-electrical odorant transduction is the lobster (*Homarus americanus*) that has two alternative olfactory signal transduction pathways. After odorant-receptor binding, adenylyl cyclase rapidly and transiently catalyses the formation of cyclic 3',5'-adenosine monophosphate (cAMP) from adenosine 5'-triphosphate as well as by hydrolysed of membrane phosphatidylinositol by phospholipase C (PLC) with the liberation of 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) in outer dendrites of olfactory receptor cells (Boekhoff et al. 1994). The cAMP pathway results in the activation of potassium channels in the

plasma membrane and hyperpolarization of the chemosensory neuron (Michel and Ache 1992). The IP3 pathway opens cation channels leading to depolarisation of the chemosensory neuron (Fadool and Ache 1992). These two second messenger pathways are linked directly to opposing outputs and can occur within the same chemosensory neuron (Hatt and Ache 1994). As natural odors are usually complex blends of chemical molecules they probably activate both cAMP and IP3 pathways with different odorants eliciting opposing responses in an individual neuron, and as a single chemoreceptor neuron may have more than one receptor type, a single chemosensory neuron could function as a complex integrating unit (Cromarty and Derby 1997, Kreiger and Breer 1999). Detailed knowledge of the relevant receptor types and transduction elements, such as that described for the lobster, facilitates efforts to find chemical molecules that activate the olfactory system of Crustacea and assist in the development of manufactured attractants which might be attractive to them and entice them to a point source, such as a trap. Indeed, based on the knowledge of chemoreception in insects, pheromone baited trap technology is being developed to control, in a species specific and ecologically sustainable manner, agricultural pests (Howse et al. 1998, Ebbinghaus et al. 2000, Vlieger 2000).

The axons from the bipolar chemosensory neurons of antennular aesthetascs join to form the lateral aspect of the antennular nerve, the axons of which project exclusively to the columnar neuropil of the olfactory lobe of the brain (Sandeman and Luff 1973, Sandeman and Denburg 1976, Schmidt and Ache 1992, Sandeman and Sandeman 1996). There are thousands of neurones in the olfactory lobe. For example, there are approximately 200,000 neurons in the olfactory lobe of the crayfish (*Cherax destructor*), which has 30-40% of its brain volume devoted to the processing of olfactory information, and hence recording from within it will result in a field potential generated by a large number of chemosensory neurones (Sandeman and Sandeman 1998). The glomeruli in the olfactory lobes act like neural junction boxes linking the projections from receptor neurons and relay information about the odor to the olfactory lobes. The olfactory lobe is also closely related to other structured neuropil in the brain called the accessory lobe and connected by the olfactory-accessory tract (Ache 1982). The olfactory-accessory lobe complex is connected to neuropils in the protocerebrum, the medulla terminalis and the hemi-ellipsoid body by the olfactory-globular tract (Sandeman et al. 1993). Comparisons for sexual dimorphism in the size of the olfactory lobe between the sexes can offer insights into the relative olfactory processing capability of an animal (Beltz et al. 2002).

ELECTROPHYSIOLOGY OF CHEMORECEPTION

In the *Drosophila* fly, the olfactory system contains approximately 10,000 receptor neurons (compared to 100,000,000 in the human) (Warr et al. 2001). Partially due to

the low number of chemoreceptors in *Drosophila* olfactory function can be analysed in vivo by measuring the electrophysiological response by single-unit recording (Boeckh and Ernst 1987). The relatively large size and accessibility of the antennules in large decapods, including the lobsters *Panulirus argus*, *P. californicus* and *Homarus americanus*, the crayfish *Austopotamobius torrentium* and various crabs, such as *Callinectes sapidus*, has led to them being used as model systems for single-unit patch-clamp recordings to study the cellular electrophysiology of chemoreception to feeding stimulants (Johnson and Ache 1978, Derby and Atema 1988, Grunert and Ache 1988, Gomez et al. 1994, Derby 1995 and Cromarty and Derby 1997). Electrophysiological measurements are made in such studies by placing an electrode into the base of the sensillum and then recording neuronal activity following odor stimulation (Kaissling 1995). The electrode should ideally only record from one neuron, although recordings from immediately adjacent neurons are also possible but limited as multi-neuron recordings may be too complex for analysis (Mellon et al. 1992, Shanbhag et al. 2000). Nevertheless, an alternative approach for electrophysiological studies is to focus on bundles of olfactory neurons (Leise and Hadfield 2000). The individual axons in the antennular nerve are extremely small, about 0.3 micron in diameter, and form tight bundles and hence single unit recording is not readily achievable on small decapods, such as penaeid prawns (Laverack 1975). In addition, due to the extremely small diameters and large number of neurones, it is extremely difficult to obtain electrical recordings from individual axons without destroying them. This problem can be overcome by recording from a large number of single neurones. In insects, it is possible to record the summed DC potential responses of thousands of olfactory neurons from their antenna and represent the whole antennal response to odorants. These are known as electroantennograms (Park et al. 200). The advantage of this method is that the electroantennogram can register responses to a wide variety of odorants and they have been successfully employed in insects for pheromone identification (Cosse et al. 1997). In a similar way, recordings can be made outside of the antennule but within the olfactory neural pathway.

Electrophysiological recordings have been made from the olfactory-accessory tract itself and the olfactory lobe in *Panulirus argus* and *C. destructor* (Maynard 1966, Sandeman and Sandeman 1998). Based on local field potential recordings in *Panulirus argus* it has been shown that there are at least two classes of chemosensory interneurons that ascend the olfactory-globular tract, one is also responsive to tactile and visual stimulation, while the other is only chemosensory (Ache and Fuzessery 1979). Similarly, as the olfactory-globular tract connects the olfactory-accessory lobe complex partially through the medulla terminalis, which is located in the eyestalk itself, the olfactory-globular tract is part of the optic nerve and accessible for electrophysiological recording (Ache and Fuzessery 1979). Recordings have also been obtained from the hemi-ellipsoid neuropile of freshwater

crayfish that are postsynaptic to the olfactory neuropile projection neurons (Mellon et al. 1992).

WHOLE ANIMAL BIOASSAY

The purification of a reproductive pheromone requires a bioassay and is critical in the effort to isolate the specific molecules for later chemical characterization. Ideally some form of experimental behavioural whole animal bioassay is required to demonstrate that a pheromone is present in the environment to trigger attempts to purify the pheromone. In other studies on crustacean pheromones the bioassay has a behavioral component, such as odor tracking or specific behavioural displays that identify when the chemical signal is present (Dunham 1988). However, the bioassay response must be controlled for contributions made by other sensory inputs. Difficulties also arise if additional stimuli must be present for the full expression of the whole organism response to the putative pheromone. The most effective way to explore the role of chemical stimuli in reproductive behaviours is to begin with a sound understanding of the reproductive biology and habitat preferences of the species in question (Borowsky 1991). Only in this way can experimental methods be designed to mimic the species habitat to permit the organism to respond as naturally as possible when exposed to putative pheromones.

Three primary behavioural responses to reproductive pheromones have been identified; chemokinetic, chemotaxic and releaser reactions (Dunham 1978). Chemokinetic reactions include the speed of movement (orthokinesis) and the frequency of turning (klinokinesis) whereas chemotaxic reactions are movements directly towards or away from the pheromone source. Releaser reactions are typically stereotyped responses, such as courtship, mating, etc, elicited by the pheromone. A primary difficulty in the demonstration of reproductive pheromones is the unreliability of the bioassays employed and their ability to discriminate between a specific reproductive pheromone and other non-pheromone chemical signals, such as food, as well as those associated with tactile and visual cues (Hardege et al. 2002). An additional difficulty is the standardisation of the biological status of the animal receiving the pheromone signal. For example, in crabs the male is not continuously receptive to a female pheromone (Bamber and Naylor 1996a). The animal must also be capable of carrying out the sophisticated behavioural strategies needed to track the scented plumes to their source (Atema and Voigt 1995; Atema 1998). The development of an aquarium system that allows the complete expression of all chemokinetic, chemotaxic and releaser behavioural responses is extremely difficult to achieve with the primary one being confinement of the animal within a limited area. In addition, the chemokinetic and chemotaxic behaviour of animals, such as prawns, under control conditions may be so variable as to mask any change in the behaviour that is associated with that induced by a pheromone. In contrast,

releaser behaviour responses typically produce a stereotype signature response, such as a courtship display or mating.

The most robust releaser behavioural response in Crustacea is the stereotype courtship found in crabs of the families Cancridae and Portunidae. In *Callinectes sapidus*, the male, upon an encounter with a pre-moult female, performs a stereotyped dance display including rising up on extended pereopods and protruding chelipeds. Afterwards the male commences a slow forward search motion and 'captures' the female, termed pre-moult cradling, and carries her around for several days until she moults (Hartnoll 1969). This behavior is distinct from feeding and normal locomotor movements and hence is a robust behavioural assay (Bamber and Naylor 1996). In crabs, the capture of the female by the male may occur up to 8 days before she actually moults (Teytaud 1971). Pre-moult females already carried by a male do not elicit a response from other males. Copulation is restricted to a brief period immediately after the female moults after which the female continues to be carried around by the male for several days before she eventually escapes. Even though this behaviour is a robust stereotyped behaviour there is nevertheless some variability, including abbreviated interactions and occasions when the male dance display is not always elicited (Christofferson 1970).

Another approach to a whole organism bioassay for investigation into pheromones is to examine odor tracking behavior. Odor-mediate searching is determined by three primary factors: the chemical composition of the odor, the release rate of the odor and the physical hydrodynamics. The chemical composition of feeding attractants for Crustacea has been extensively studied and been found to be relatively common metabolites (Finelli et al. 2000). In contrast, reproductive pheromones are assumed to be species-specific and unique molecules (Bentley and Watson 2000). The chemical identity of reproductive pheromones from Crustacea is largely unknown and hence reproductive pheromone tracking behaviour studies must be based on estimates when such pheromones are being released from an animal. The only factor that an experimenter can have a significant degree of control over is the development and design of a testing tank or arena with specific, and hence reproducible, hydrodynamic attributes.

In the aquatic environment mass flow characteristics are critical in chemical communication. Chemical attractants, including feeding stimulants and pheromones, are transported from the point of release from high to low concentration via molecular, advection, and turbulent diffusion (Weissburg 2000). After release from a point source undiluted chemical packages can be transported in organised fluid tracks in the form of vortices of rotating parcels of fluid (Doall et al. 1998). A pheromone may be carried as a turbulent odor containing discrete odor eddies (vortexes) separated by clean water and operate over relatively long time scales

that animals may use for orientation, or chemotaxis. Such vortexes may develop down stream of large objects (>1cm in diameter) in slow flows (0.1-1 cm/s) or of small objects (100 micron diameters in fast flows (10-100 cm/s) (Zimmer and Butman 2000). The efficiency of chemotaxis is dependent on the hydrodynamics of the environment and on the pheromone itself being carried by both advection and turbulent mixing. In the absence of either there is no directional information.

The magnitude of turbulence covaries with flow speed, surface roughness and animal size (Zimmer and Butman 2000). Turbulent mixing dilutes waterborne chemical stimuli as well as creating patchiness in chemical distribution. Although water flow speeds may be necessary for orientation, with flows of 1cm/s producing optimal smooth-turbulent boundaries for directional information, high water flows (>4 cm/s), induce rough turbulent boundary layers with an associated lost of directional information (Weissburg 2000). Directional information from an odor plume is unlikey in wave swept habitats as current speed and direction changes on a time scale of seconds (Denny 1988). In contrast, tidal flow is more constant and consistent with changes in flow direction occurring over hours (Pickard and Emery 1990). Although the pheromone may be the chemical stimuli inducing a behavioural change in an organism, it is the water flow that provides directional information (Weissburg and Zimmer-Faust 1993). Overall, effective odor tracking depends on appropriate flow. When flow is slowest, turbulent mixing is minimal and a strong odor signal is present but with little directional information. On the other hand, at high flow there is increased turbulent mixing and dilution of the signal strength while at the same time having significant directional information. Hence the hydrodynamics of pheromone transport can have a profound influence on sensory and behavioural mechanisms of receptive organisms. As prawns inhabit the benthic zone in areas of tidal influence hydrodynamic conditions are favourable outside of slack tide for directional information to be obtained from odor plumes to locate its source.

A large variety of tank designs for creating and characterizing specific field flow environments for marine chemical ecological studies have been utilized. Large flumes, wave tanks and Coutette flow cells have been designed to produce one-dimensional, unidirectional, turbulent, oscillatory or laminar-shear flows (van Wazere et al. 1963, Vogel and LaBarbera 1978, Muschenheim et al. 1986, Nowell 1987 and Miller et al. 1992). These tank designs have been developed for examining specific aspects of laminar and turbulence water flow. A further modification of these designs includes those for examining for the presence of chemical stimuli in the environment and the behavioural response on an organism or 'olfactometer assays' (Kennedy 1977, Borowsky 1985). Typically these tanks produce a turbulent flow down a raceway. The source of the pheromone is introduced at one end, usually through channels or a maze, so the organism that is odor tracking must make a choice on one particular channel. If the animal is odor tracking then it will follow

the trail into the appropriate channel. Although the tank design is critical in experiments on the response of marine organisms to chemical stimulated responses, the natural behaviour and life history of the organisms is critical to meaningful results. For example, several apparently well designed experiments, with respect to the control of various variables, failed to conclusively demonstrate that premoult female amphipods released a pheromone that attracts males from a distance (Ducruet 1973, Hammoud et al. 1975, Hartnoll and Smith 1980). However, as demonstrated for other amphipod species, amphipods typically only express many behavioural tracts, such as searching in the open, during hours of darkness (Borowsky 1991). The experiments referred to above that did not demonstrate the presence of mate searching behavior took place during hours of daylight and hence may have resulted in false negative findings as the search behavior was inhibited by light. Similarly design problems for an effective 'olfactometer' assay exist for other species of Crustacea as some species do not adapt well to laboratory conditions which hampers efforts to describe the repertoire of their typical behavioural responses to the stimuli and subsequent indication that a pheromone is actually present (Borowsky 1984).

Methods

MODEL SPECIES

Adult black tiger prawns (*Penaeus monodon*) were originally used as one of the model species. Researchers at AIMS have worked on this species for several years and have the capacity to maintain this species in captivity and regularly hold several hundred adult and juvenile *P. monodon* at any one time. However, the commercial wild prawn fishery sector does not consider *P. monodon* as being of any significant importance to their fishery. Nevertheless, it is potentially a valuable model species. There is also the potential for a non-trawl capture method for adults of this species to supply the broodstock requirements of the prawn aquaculture sector. The black tiger prawn was used in preliminary studies, especially for the development of the 'olfactometer assays' (Kennedy 1977, Borowsky 1985). However, due to their high cost ($125-150 per prawn) and high mortality of adults when held in captivity for periods of several months this species was regarded as unsuitable for the pheromone purification trials.

Alternative model species included the brown tiger prawn (*P. esculentus*) and the green, or grooved, tiger prawn (*P. semisulcatus*). Together they form the most valuable prawn trawl sector (Williams 1997). These two species were sourced from the Townsville region. Their behaviour is different from that of the black tiger prawn as these species remain burrowed in sand/mud during the day, whereas *P. monodon* does not, and are only active during the hours of darkness. They potentially offer a good model for which to demonstrate behavioural changes due to pheromonal

influence. After initial tests on both species we selected *P. semisulcatus* as the experimental species on which to concentrate research efforts.

The original proposal also recommended that the Ornate rock lobster (*Panulirus ornatus*) be considered as a potential experimental species as it has favourable characteristics for whole animal bioassay experiments. Adults are large and can be readily tracked and individuals identified under video surveillance. Methods to collect undiluted urine from unrestrained adults have been developed and it is believed that this would increase the likelihood of isolating the chemical nature of pheromones released by Crustacea (Breithaupt et al. 1999, Lindstrom 1991). However, comments from trawl operators indicated that this species was not appropriate given the main objective of the project – the investigation of the possibility of using pheromone baited traps for penaeid prawns – so no trials were made using this species.

NEUROBIOLOGY OF CHEMORECEPTION

Chemoreceptors

A method was devised in which the aesthetasc sensilla could be removed from the olfactory organ, leaving the cuticle and the bases of the sensilla intact. Prawns were anaesthetised in seawater and ice slurry then killed by heart puncture and severing of the ventral nerve cord. Antennules were excised at the proximal basal segment and immediately placed into 10% formalin, 0.1 M phosphate buffer saline (PBS) at pH7.2. Antennules were stored for approximately 24 hours at 4ºC before antennules were washed and immersed in 0.1M PBS. The outer flagellum was excised from the basal segments and distal portion of annuli not containing the aesthetasc rows. A blunt brush was used to carefully remove all the aesthetasc sheaths leaving only the antennule base. The lateral carapace containing the remaining aesthetasc bases was then 'filleted', teasing the carapace from surrounding epithelial tissues. Carapace fillets were subsequently dehydrated in an alcohol series (10 min each in 70, 80 90 and 100% Ethanol) to Xylene (2 min) then permanently mounted on slides using MountQuick (ProSciTech). Accurate counts could then be made under the light microscope and a digital imaging system. Digital images of all aesthetasc fillets were recorded with a light microscope, analog black and white camera and Apple computer software (BTV Pro). Image J 1.32 (Wayne Rasband, NIH) was used on scaled images of aesthetascs to record the length of aesthetasc rows, counts of aesthetascs per row and number of rows. These were then used to calculate total aesthetasc rows per antennule and total aesthetascs per antennule. Preparations were made of the left and right antennules of both sexes from prawns ranging in size with a carapace length between 41 mm to 68mm.

A total of 8 pairs of female and 3 pairs of male antennules were compared, recording the total number of aesthetasc rows per antennule, total aesthetasc counts, average length of aesthetasc rows and the average number of aesthetascs per row. The latter two measurements for each pair were compared using a paired t-test. The allometric equation, $Y = aX^b$ (Flores and Negreiros-Fransozo, 1999) was used to estimate the allometry of aesthetasc measurements. The relationship was linearised to the following equation, $\log Y = \log a + b \log X$. An analysis of covariance (ANCOVA) was used to describe whether sex differences existed in aesthetasc measurements. Two factors were assessed for significance, carapace length and sex. Carapace length was used as a covariate in the assessment of a sex difference in the relative variables, and was also used to determine whether intraspecific variables varied allometrically. A significant effect in the interaction term (i.e between sex and carapace length) indicates a significant difference in the slopes of the lines, whereas a significant effect in the "sex" factor indicates a significant difference in the intercept.

Electrophysiology

Electrophysiological procedures were directed at recording electrical potentials associated with the activation of the olfactory receptor organs by possible pheromones and attractants. It is important to stress, however, electrical activity in the small input fibres of the crustacean olfactory receptor neurons is, for purely physical reasons, extremely difficult to detect. Electrophysiological recordings from neurons in the antennular nerve were attempted from isolated antennules. After killing the prawn, following immersion in ice cold seawater, an entire antennule and part of the head was removed from the animal and pinned down in a dish. The antennule was perfused with warm saline based on the method first developed for the crab (Sandeman 1967). The exoskeleton over the flexor and extensor muscles of the flagellum was carefully removed and suction micro-pipettes applied to the teased antennular nerve. Several electrode tips were tested but all were variations on a fine-tip glass electrode, filled with 3 M - potassium chloride (KCl) solution with a resistance of approximately 80 MOhms (Sandeman and Sandeman 19998). One electrode was inserted into the base of the remnant eyestalk and served as a reference electrode. Another microelectrode was mounted on a micromanipulator and driven down through the antennule to a depth of a few microns. The antennular nerve is invested with a substantial sheath that has to be penetrated with the microelectrode tip before the axons can be reached. Once within the antennular nerve sheath axons could be impaled and recorded. Recordings were digitised, analysed using Scope software (PowerLab ADInstruments) and files stored on an Apple labtop computer. The effects of attractants, including solutions of 1 to 100 micromolar glycine, taurine and betaine, known to have resulted in changed patterns of electrophysiological recordings, were tested as positive controls (Carr and Derby 1986a).

Pheromone source - rosette glands

As describe in the introduction, rosette glands have been found close to the openings of the green glands (antennal glands) in lobsters and have been associated with the excretion of pheromones. The existence of rosette glands associated with the antennal glands was therefore examined in prawns. The anterior part of the head including the base of the antennae and antennules were dissected and fixed in situ in Davidson's Fixative (In 1 litre: Formalin 220 mL, 95% Ethanol 330 mL, distilled water 335 mL; glacial acetic acid 115 mL). After 24 hours fixation, tissues were processed for routine histology by removing wax, dehydrating tissue and embedding in wax. Serial 10 micron sections were cut dorso-ventrally through the base of the antennules around the region of the nephrophopore. Sections were stained using Mayer's Haematoxylin and Eosin (H&E). Sections were examined for the presence of rosette glands.

Whole animal bioassay – odor tracking

Wild adult *P. semisulcatus* were supplied by trawlers operating off Cairns and either shipped by road or air freighted to the Tropical Aquaculture Facility (TAF) at the Australian Institute of Marine Science (AIMS), Cape Ferguson (19°16' S). Up to 30 prawns were held in a circular 4 m diameter (12.56 m^2 floor area) holding 8,000 L of seawater. Seawater was obtained from a pipeline reaching 200 m off-shore, with an intake head at 3 meter depth, and pumped to two 450,00L storage tanks, where the water is settled for approximately 24 hours. Post-settled water is gravity fed to additional 100,000L settling tanks and held for approximately 8 to 12 hours and is finally pumped from just below the surface level through a manifold of nominal 10 and 5 µm cartridge filters before passing into the mariculture unit.

The seawater in the prawn holding tanks is partial recirculating, at 2,400 L/hr with each pass being processed through a counter-current ozone Venturi protein skimmer (foam fractionator), through a tickle down bio-tower reactor and followed by an ultraviolet sterilizer at 254 nm with a nominal rating of 2.5 µW sec/cm^2. In additional tanks received a 200% water exchange per day. Tanks were illuminated with incandescent lamps with an average daylight intensity of 2 lux at floor level. Prawns were fed twice daily on a diet of squid (Loligo spp.), New Zealand green lipped mussels (*Perna canaliculus*) and pipis (*Plebidonax* spp.) at a rate of 4% of average body mass per day. Water, temperature and salinity varied between 24°C and 30°C and 33 to 35 ppt, respectively, over the study period. Throughout the experiment prawns were monitored daily for moult.

This objective required development of a bioassay to detect pheromones released by prawns followed by development of a suitable method to collect chemicals secreted by prawns. The whole animal bioassay, or 'olfactometer assay', were performed in a

specially designed aquarium tank (Figure 1.2). Male prawns could choose between four water types that entered the main tank arena through four channels. In essence seawater enters one of four 40 litre holding tanks containing females in various physiological states. Seawater was pumped into a 900 L storage tank (tank 1) where it passed through a large capacity protein skimmer processing 60 L/min. It then passed into a second storage tank (900 L, tank 2). From there a portion of the water was then pumped back into the first tank at 20 L/min. A recirculating system was designed to remove as much particulate matter from the water as possible. The water from the second storage tank was then pumped to a second protein skimmer and processed at 12 L/min. The water passed to a third sump tank (100 L, tank 3) where it was pumped through three activated carbon filters to remove dissolved organics. The seawater was held in a header tank (tank 5) where it was pumped/ siphoned into each female holding tank (40 L) at 0.86 L/min. The overflow from the female holding tank was directed into its corresponding chamber in the arena. Excess water from tanks 3 and 5 was then directed to a storage tank (100 L, tank 4) where it was pumped into each chamber within the arena at a flow rate of 1.5 L/min. This was introduced into the tank via small microjets to direct water flow out of the chamber and into the main area of the arena (Figures 1.3 and 1.4). Resident time of the seawater in each female tank was between 20-40 minutes before overflowing into one of four channels of the main arena tank. The main arena tank housed 3 to 6 male prawns.

The prawns were held on an artificial photoperiod of 14 hours light and 10 hours of darkness. Light during the day was supplied by 4 fluorescence overhead lamps (Fig. 3a). The arena was illuminated at night by 4 far-red fluorescence lamps, which have a luminance maximum at 660 nm. The peak photosensitivity of prawn eyes occurs between 520 and 545 nm. Filming under read light allowed observations to be made of prawns in the tank as their visual senses would not have detected 'light' (Fig. 3b). Based on detailed behavioural observations the prawns behaved under far red-light as if they were in darkness, they emerged from burial and began to be active when the white lights were turned off and the far red lights were turned on. The behaviour of these prawns were monitored by an black and white half-inch CCD analogue video camera processed through a digital converted to Apple Macintosh computer software for storage (BTV Pro) and processing imaging (Image J, NIH) (Figure 1.5 and 1.6). All filming was done during the prawns' night when they are naturally active.

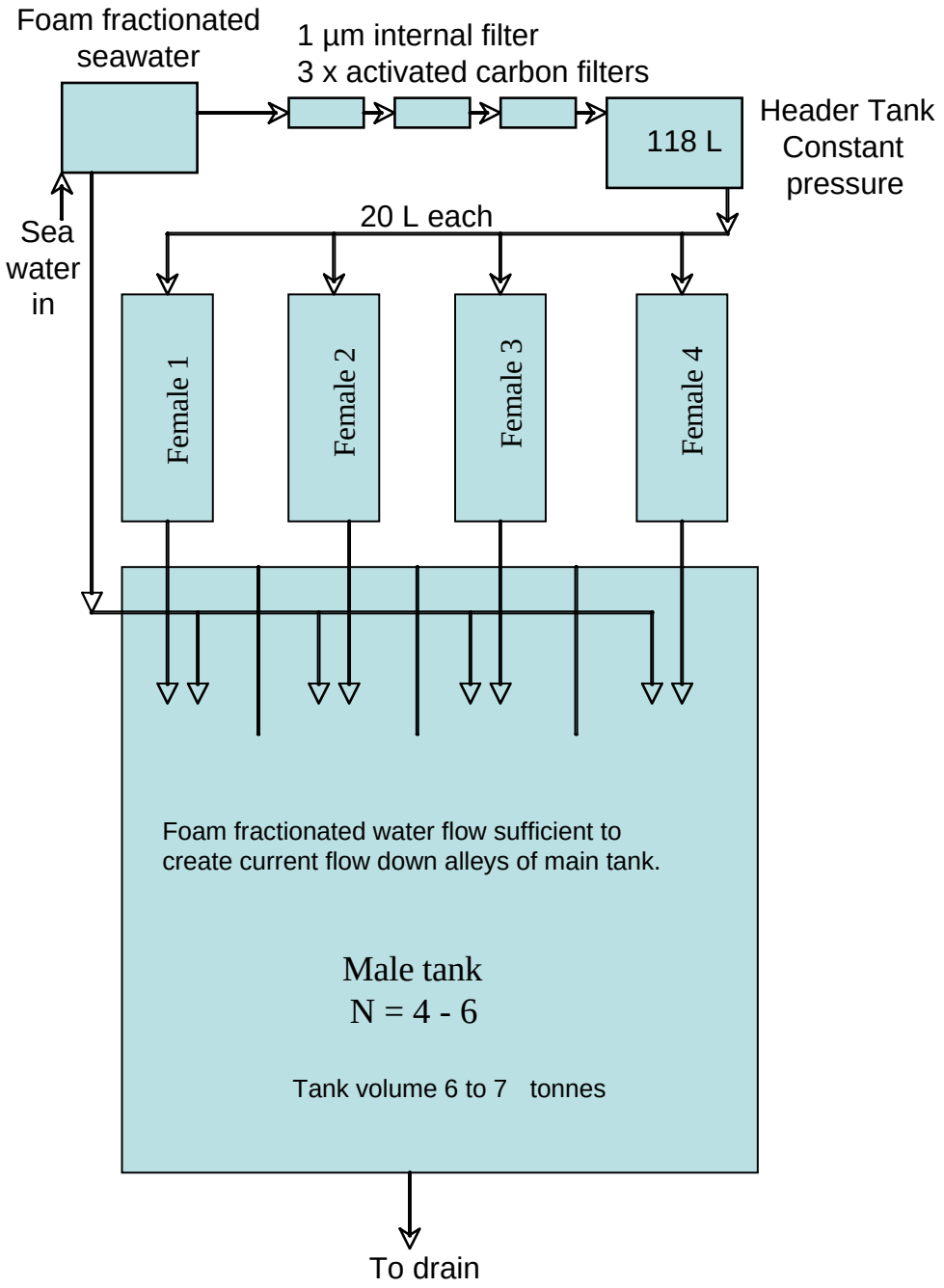


Figure 1.2. Schematic overhead view of water treatment and 'olfactometer' aquarium tank for video surveillance of prawn behavior and response to putative pheromones.

	Total
Area of arena	9.48 m^2
Volume of arena	5.69 m^3
Protein fractionator a	60 L/min
Protein fractionator b	12 L/min
Channel flow rate (1.50 L/min)	6.0 L/min
Female tank flow rate (0.9 L/min)	3.4 L/min

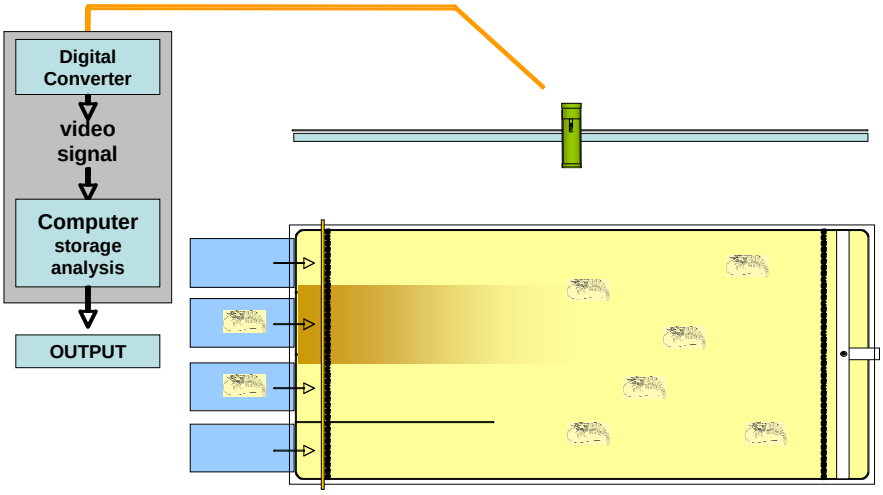


Figure 1.3. Schematic view of 'olfactometer' aquarium for testing for the presence of female pheromone released into a flow through channel and into the main arena area containing males with overhead video monitoring.

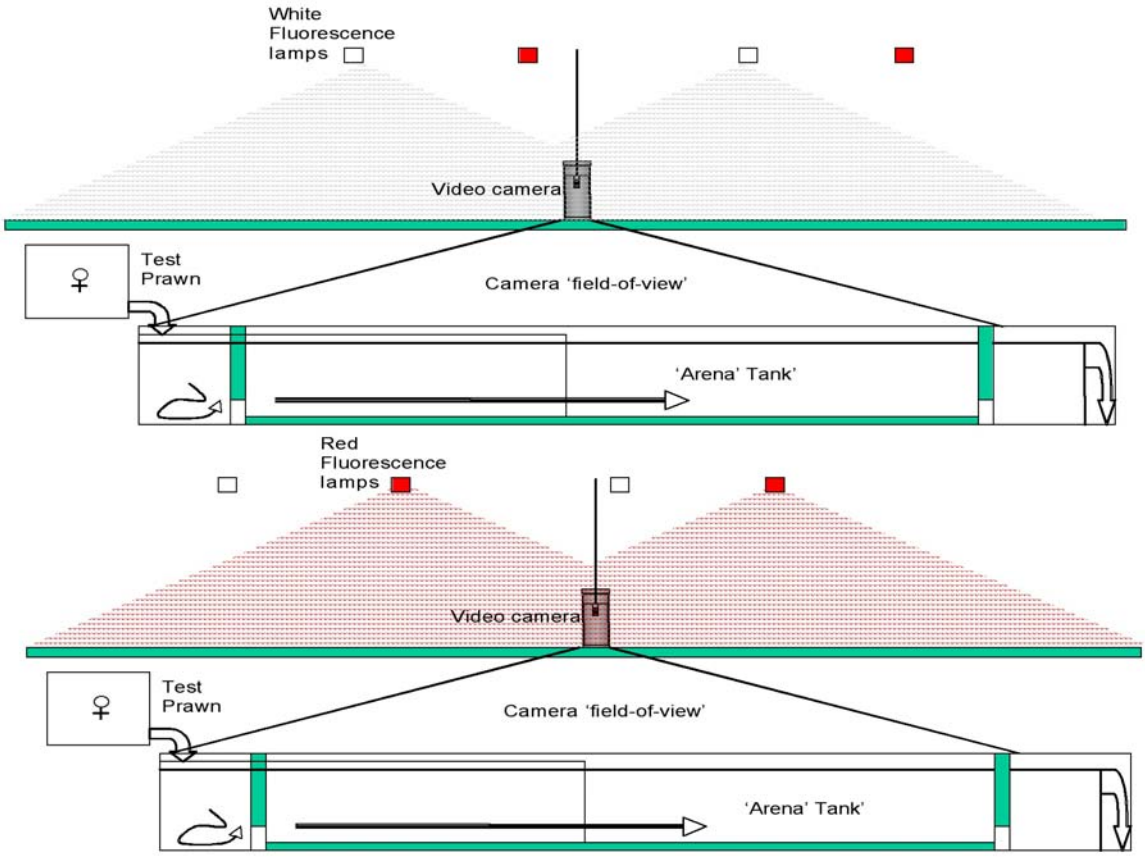
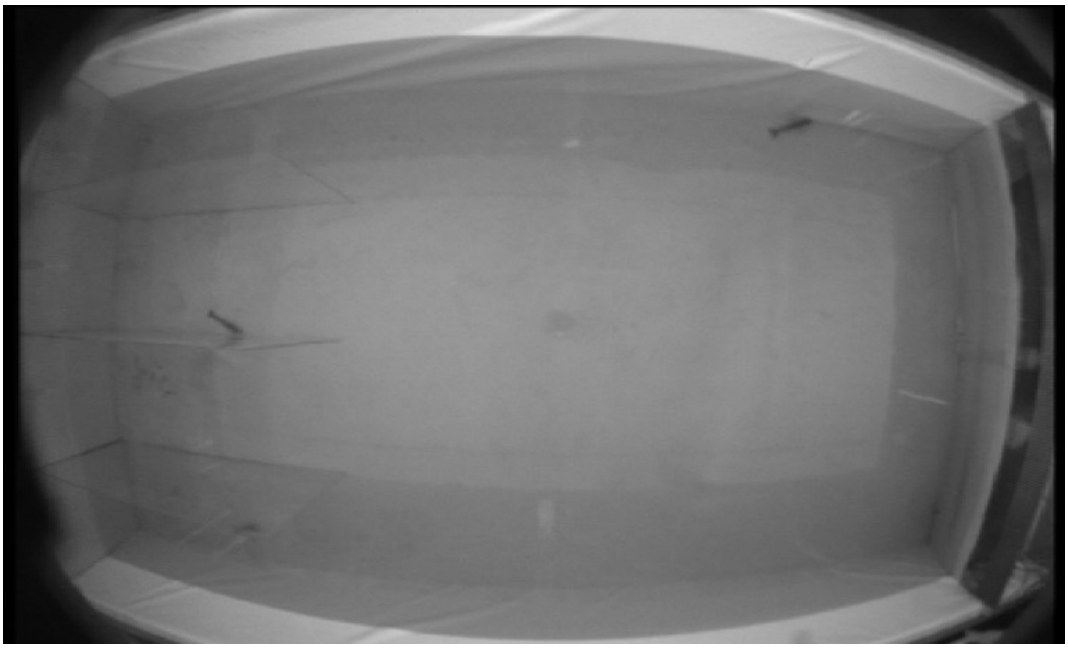


Figure 1.4. Aquarium set-up for 'olfactometer' assay. During the day white fluorescence lamps provided illumination (a) while at night red fluorescence lamps were used (b) to give sufficient light for video surveillance. Green bar represents white diffuser ceiling. Female holding tank at far left. After turbulent mixing the water flow leaving the female holding tank flowed down one of 4 channels. See Fig. 1.3 for overhead view.



Surveillance Camera (analog)

Figure 1.5. Analogue video output from overhead camera of 'olfactometer' aquarium tank. Four channels, separated by clear Plexiglas walls can be seen on the left. Female prawn holding tanks out-of-view on far left. Water exit over weir on far right. Three prawns can be seen in the tank. Image is distorted as a wide angle lens had to be used to view the entire arena area.

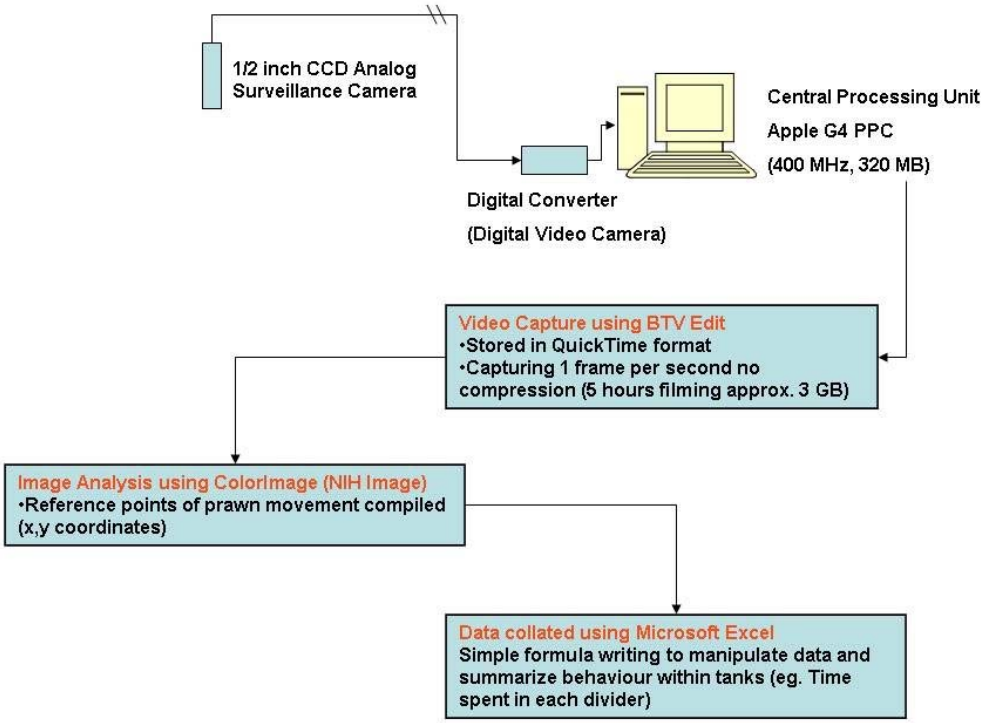


Figure 1.6. Video camera monitoring and computer assisted motion analysis system.

For data analysis video footage of the tank was captured at 1 frame per second. The film was divided into 1500 frames (1500 seconds or 25 mins). The footage was imported into Image J where the prawns were tracked by recording the X Y coordinates of prawns every second over time. The raw data was transferred to excel and added to make 3000 frames (50 mins). Traces were created of prawns movements. Hot spot graphs were created by dividing the tank into a grid of 48 x 25 (1200 locations). XY coordinates were summed for each location on the grid and graphically represented as a change in colour (Fig. 1.21). Information regarding changes in behaviour and hot spots in activity was collected.

The aquarium tank arena for behavioural bioassays was designed not only for video surveillance but also with the capability to collect seawater samples for pheromone analysis (see Objective 2). Seawater was pumped into a 900 L storage tank (tank 1) then through a large capacity protein skimmer processing 60 L/min. It then passed into a second 900 L storage tank (tank 2). At this point a portion of the water is then pumped back into the first tank at 20 L/min. The recirculating system was designed to remove as much particulate matter from the water as possible. The water from the second storage tank was then pumped to a second protein skimmer and processed at 12 L/min. The water passes to a third 100 L sump tank (tank 3) where it is pumped through to a carbon filtration unit, identical to the one described in the previous system. The seawater was held in a header tank (tank 5) where it was pumped/siphoned into each female holding tank (40 L) at 0.86 L/min. The overflow from the female holding tank is directed into its corresponding channel in the arena. Excess water from tanks 3 and 5 was then directed to a storage tank (100 L, tank 4) where it was pumped into each channel within the arena at a flow rate of 1.5 L/min. Incoming water was introduced into the tank via small micro-jets to direct flow out of the channel into the main area of the arena.

After construction the 'olfactometer' tank the system was commissioned by following 3 male prawns in the presence or absence of a food source as the chemo-attractant. Prawns were video surveillance throughout the hours of darkness under far-red light and the data recorded as a digital computer file. Each file was reviewed the following day for signs of odor tracking behaviour.

It was not possible to use computer software to automate tracking of the prawns for several reasons. Firstly, the prawns in relation to the size of the tank were a small point source and computer programs designed to track a moving object rely on the object being of significant size in relation to the arena being monitored. The aquarium tank was designed to be as large as possible for the prawn in an attempt to procure 'normal' or natural behavior from them. In terms of tracking the animals two problems became apparent. The use of red light did not provide sufficient contrast in the corners. When the image was processed (threshold function in Image)

for tracking the prawns were indistinguishable from the background (untrackable). In addition the tracking programs rely on individual prawns being recognisable at all times. The prawns' behaviour meant that they often crossed over one another and were not distinguishable as two objects. Essentially the surveillance software was losing track of the animals. This necessitated the selection of only small amounts of digital video recording to computer analysis. At the end of each night of recording the video was reviewed and only those recordings that appeared to have significant changes in behavior were analyzed. At least one recording of controlled behavior was analyzed for comparison. It soon became apparent that the recordings from the 2 outer channels were heavily biased and could not be used as a result of prawns tracking the walls of the tank. This was overcome by cutting the number of channels holding females from four to two and only using the two central ones.

Trials were made with either non-moulting females (inter-moult) or immediate pre-moult females, i.e. that moulted during the night of recording. Females were held in the flow-through holding tanks with the overflow feeding into one of the channels. Due to the consistent activity of prawns around the walls of the main tank, channels 1 and 4 were not used as conduits of putative pheromones. Females being tested had their tank overflow only into channels 2 and 3 that would force the males into the centre of the arena if they were actively searching for the pheromone source. Two trials were fully analyzed by manually recording the location of 5 to 6 male prawns in specific sectors of the arena bioassay tank with a total of 3,000 to 10,800 frames at 1 frame per second analyzed over 50 minutes or 3 hours of activity. Recordings were made sequentially over two nights with the same female, the first night she did not moult while on the second night she did. The video footage analyzed was of the same time period for each night and spanned the time the female moulted. The female was held in tank 3 with over-flow of her tank water into channel 3.

Results

CHEMORECEPTORS

Fixed tissues containing the central nervous system and antenuules were analyzed by either light microscopy or transmission electron microscopy. The samples for transmission electron microscopy were processed and analyzed by Professor David Sandeman and Dr. Renate Sandeman at the University of New South Wales (UNSW).

The aesthetasc arrays on the outer flagellum of *P. monodon* antennules were structured similarly for both sexes. The outer flagellum was composed of annuli, each with a pair of parallel aesthetasc rows with larger aesthetascs ventrally (Figure 1.7). Fewer aesthetascs per row were found in proximal and distal rows for both sexes but males had significantly more then females (Figure 1.8). The aesthetacs are arranged in rows perpendicular to the length of the antennule (Figs. 1.9 and 1.10).

A summary of animals and measurements of aesthetasc arrays from male and female *P. monodon* (see Table 1.2) highlights the dimorphism between the sexes. Even though the average carapace length of sampled males was lower (see Table 1.3) all aesthetasc measurements were greater than in the females. In addition, all linearised variables were positively correlated to carapace length (Figure 1.11) and their descriptions are summarised in Table 1.4. Positive allometry was defined as a linear regression equation with a slope greater than 1. All measurements from males showed positive allometry while in females the number of aesthetasc rows and number of aesthetascs per row displayed negative allometry.

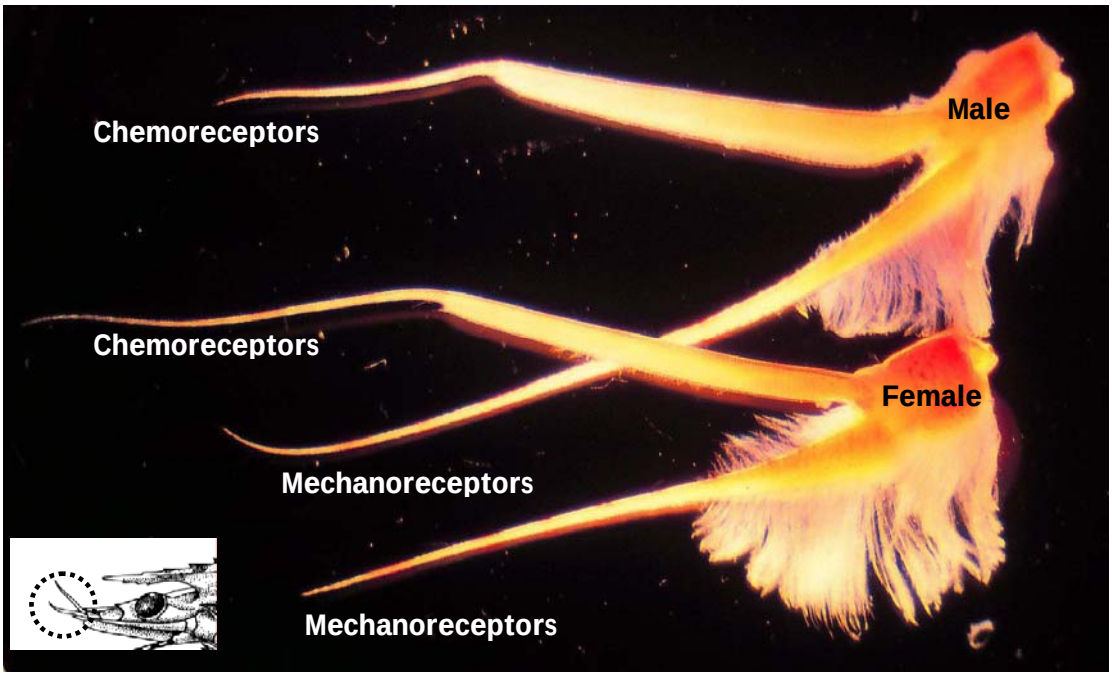


Figure 1.7. Photographs (5x mag.) showing the antennules of a male and female *Penaeus monodon*. The antennules are bifurcated flagellums. The external (upper) flagellum are coated in chemoreceptors whereas the internal (lower) flagellum are the site of mechanoreceptors.

Figure 1.8. Montage of filleted aesthetasc rows from a male (35mm CL) and female (35mm CL) *Penaeus monodon*.

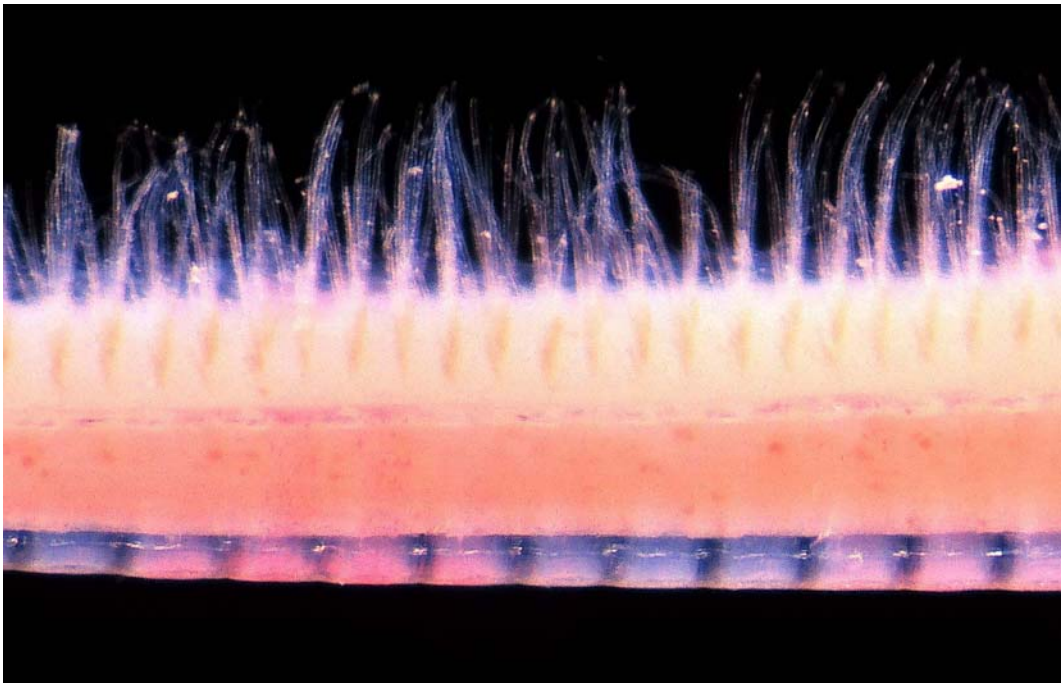


Figure 1.9. Photograph (20x mag.) showing the antennule upper flagellum of a male *Penaeus monodon*. The dorsal surface is covered in numerous chemoreceptor sensilla.

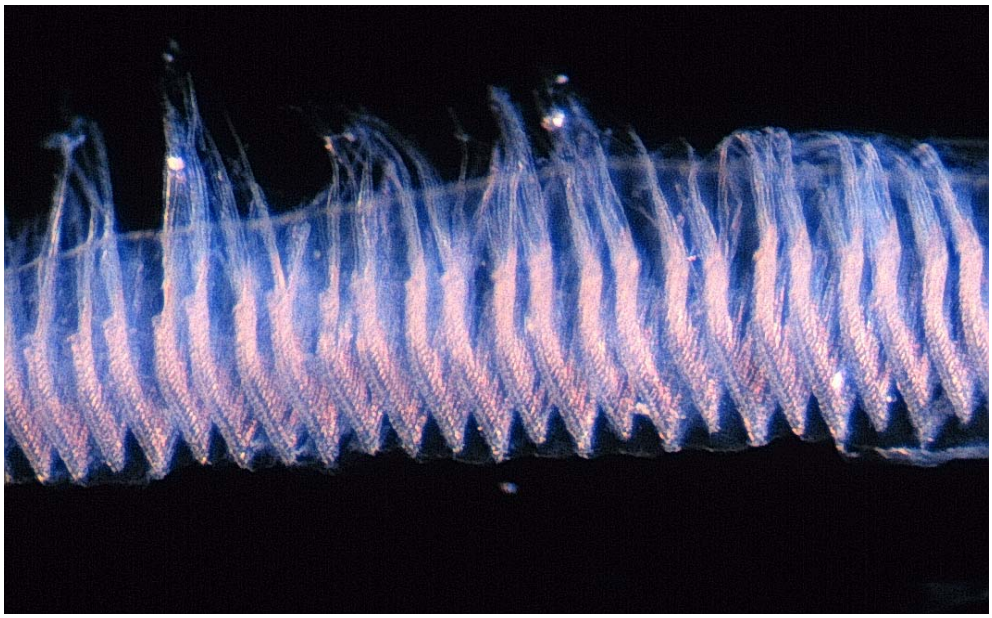


Figure 1.10. Photograph (40x mag.) showing the underside of the antennule upper flagellum that has been peeled away from the antennule. There are rows of sensilla, approximately 35 per row, and on average there are 44 rows.

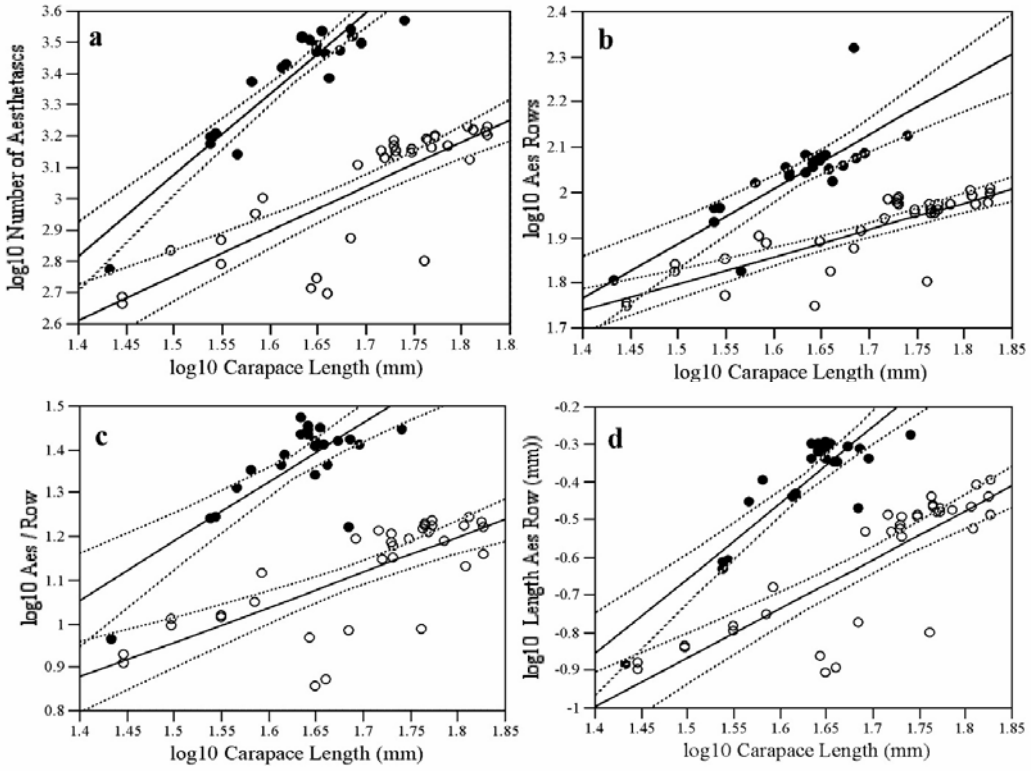


Figure 1.11. Relationship between carapace length and total aesthetascs per antennule (a), aesthetasc rows per antennule (b), average aesthetasc row length (mm) (c) and average aesthetascs per row (d). Regression analysis showing all linear relationships grouped by sex; closed circles = male; open circles= female; log indicates logarithms to base 10.

Table 1.2. *Penaeus monodon*. Sex-specific data for carapace length (CL), number of aesthetascs (Aes), number of aesthetasc rows (Aes Rows), number of aesthetasc per rows (Aes/Row) and length of aesthetasc rows (Length Aes Rows (mm)). Measurements expressed as mean ± SEM, except range in parenthesis.

	FEMALE	MALE
Number	27	23
Antennules	35	26
CL (mm)	51.38 ± 1.98 (31.4 – 67.31)	42.77 ± 1.12 (27.2 – 50.23)
Aes	1181.676 ± 73.197	2708.577 ± 151.179
Aes Rows	83.886 ± 2.428	112.308 ± 4.981
Aes / Row	14.001 ± 0.101	23.970 ± 0.165
Length Aes Rows (mm)	0.278 ± 0.002	0.426 ± 0.003

Comparison of all aesthetasc measurements between the sexes showed significant differences with results summarised in Table 1.2. A significant difference was seen in all equations when comparing the slopes as well as carapace length. Male *P. monodon* acquired more aesthetascs, more rows of aesthetasc, more aesthetascs per row as well increased length of rows with increasing body size. Most pairs of antennules from male and female *P. monodon* were not significantly different in counts of aesthetascs per row and length of aesthetasc rows (Figure 1.12). A significant difference ($p<0.05$) was only seen in the length of the rows of one female. Counts of total aesthetacs per antennule and total rows of aesthetascs per antennule for each pair were almost identical.

An individual aesthetasc protrudes from the surface of the antennule (Fig. 1.13). Individual chemoreceptors in each receptor neuron form an axonal bundle within the antennule. These eventually form the antennular nerve that projects to the central nervous system. In order to accommodate such a large number of axons in the antennule they have to be of small diameter. Transmission electron micrographs through the base of the antennules revealed large numbers of axons to be present with a diameter of between 0.1 to 0.4 microns (Fig. 1.14). The numbers of receptor neurons at the base of the aesthetasc sensilla of olfactory organs has always been a subject of speculation. From reliable counts of these from thin sections of the antennules it was found that there are between 140 and 160 olfactory receptor neurons at the base of each sensillum. Analysis of the microscopy data indicates approximately 150 chemoreceptor neurons per sensilla making an estimated 231,200 chemoreceptors per antennule or over half a million chemoreceptors per male.

The antennal nerve is a major input into the brain. The axon bundles from the chemo-receptors of the antennules terminate in the olfactory lobe of the brain (Fig. 1.15). From limited data, adult males have olfactory lobes that are considerably larger than that of females (Fig. 1.16).

Table 1.3. *Penaeus monodon*. Descriptions of allometric relationships examined. M = males; F = females; CL = carapace length (mm); + = positive allometry; - = negative allometry; log indicates logarithms to base 10; t(b=0); t-test for H0: b = 0; t(a=0); t-test for H0: a = 0.

Variable	Sex	n	Linearised Equation (log y = log a + b log x)	r^2	*t*(b=0)	Prob>|t|	t(a=0)	Prob>|t|	Allometry
Total Number of Aesthetascs per Antennule (Aes)	M	26	log Aes = -0.806448 + 2.5891927 log10 CL	0.844	11.4	<.0001	-2.18	0.039	+
	F	35	log Aes = 0.6274238 + 1.4183223 log CL	0.667	8.14	<.0001	2.12	0.042	+
Average Number of Aesthetascs per Row (Aes/Row)	M	26	log Aes/Row = -0.850651 + 1.3607476 log CL	0.608	6.1	<.0001	-2.34	0.028	+
	F	35	log Aes/Row = -0.244641 + 0.8016481 log CL	0.554	6.4	<.0001	-1.15	0.259	-
Total Aesthetasc Rows per Antennule (Aes Rows)	M	26	log Aes Rows = 0.087144 + 1.2006077 log CL	0.636	6.48	<.0001	0.29	0.775	+
	F	35	log Aes Rows = 0.89993 + 0.5988355 log CL	0.678	8.33	<.0001	7.36	<.0001	-
Average Aesthetasc Row Length (mm) (Length Aes Row)	M	26	log Length Aes Rows = -3.664511 + 2.0065486 log CL	0.774	9.06	<.0001	-10.16	<.0001	+
	F	35	log Length Aes Rows = -2.821873 + 1.3032753 log CL	0.696	8.7	<.0001	-11.07	<.0001	+

Table 1.4. *Penaeus monodon*. Univariate ANCOVA for the effects of sex on number of aesthetascs (Aes), number of aesthetasc rows (Aes Rows), number of aesthetasc per rows (Aes/Row) and length of aesthetasc rows (Length Aes Rows (mm)) with carapace length (CL) as a covariate; log indicates logarithms to base 10.

		log Aes			log Aes Rows			log Aes /Row			log Length Aes Rows (mm)		
Source	DF	MS	F Ratio	Prob > F	MS	F Ratio	Prob > F	MS	F Ratio	Prob > F	MS	F Ratio	Prob > F
log CL (mm)	1	1.2769	133.8740	<.0001	0.2574	96.1328	<.0001	0.3718	63.2800	<.0001	0.8710	116.2039	<.0001
Sex	1	3.1249	327.6218	<.0001	0.4227	157.8307	<.0001	1.2369	210.5321	<.0001	1.2660	168.9010	<.0001
Sex*log CL (mm)	1	0.1090	11.4278	0.0013	0.0288	10.7513	0.0018	0.0249	4.2303	0.0443	0.0393	5.2464	0.0257
Error	57												

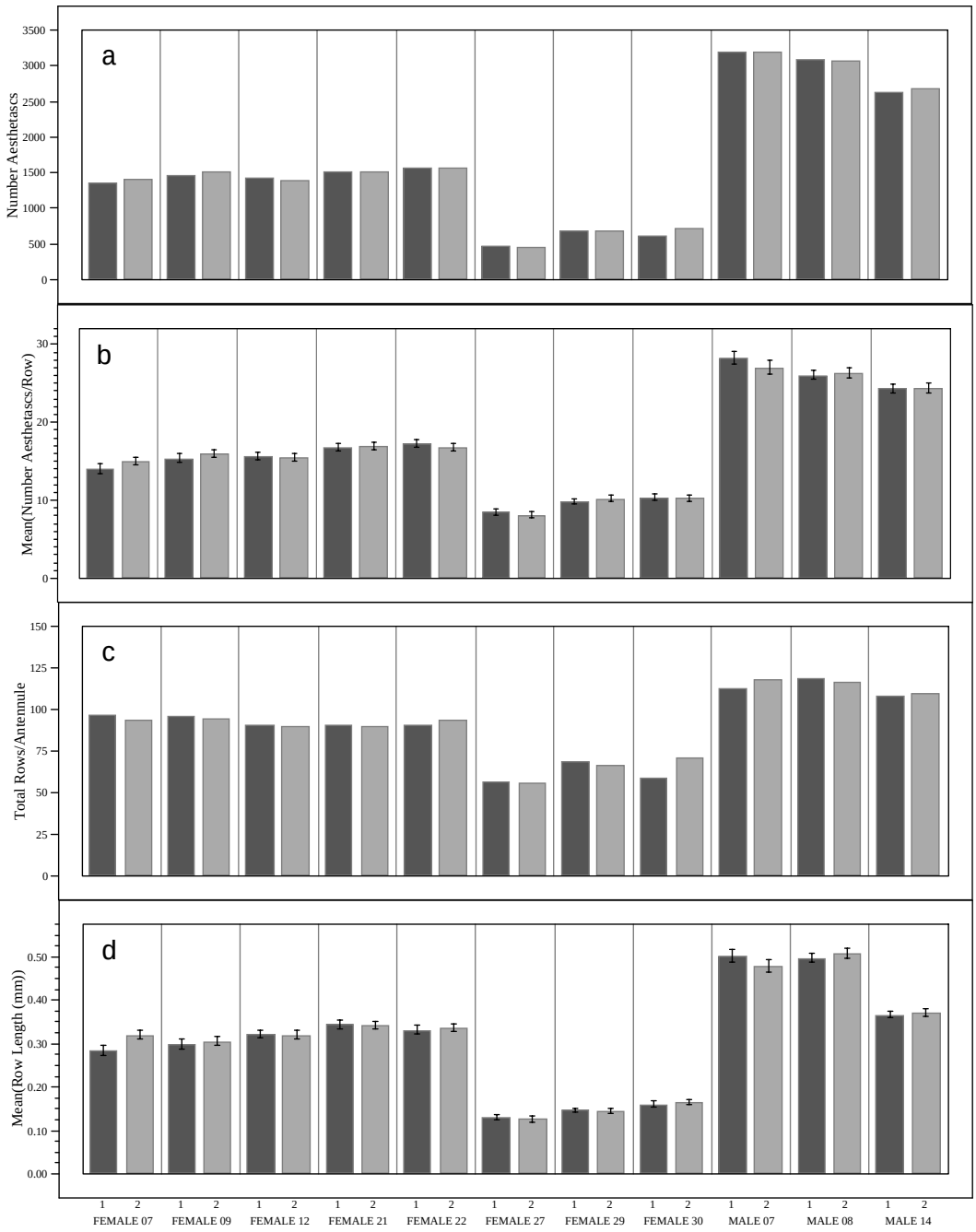


Figure 1.12. Analysis of paired antennules (1 and 2) from male (n=3) and female (n=8) *Penaeus monodon*. Number of aesthetascs per antennule (a), mean (± standard error) number of aesthetascs per row (b), number if aesthetasc rows per antennule (c), mean (± standard error) length of aesthetasc rows per antennule (d).

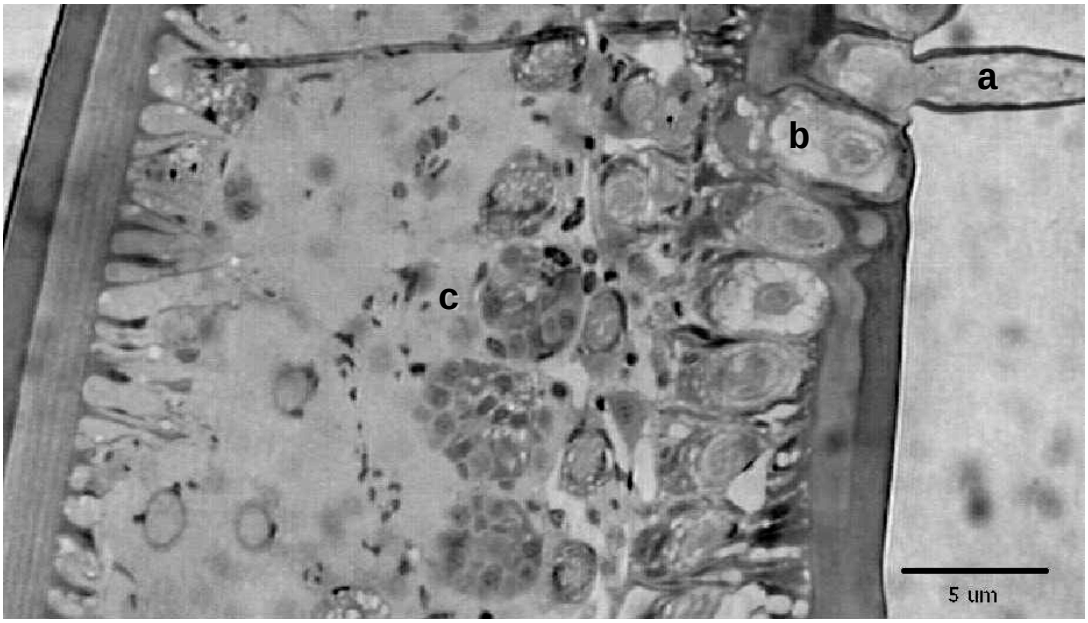


Figure 1.13. Vertical Section through the antennule of *P.monodon* showing an aesthetasc (a), dendrites (b) olfactory receptor neuron bundle (c) that will join with others to form the antennal nerve.

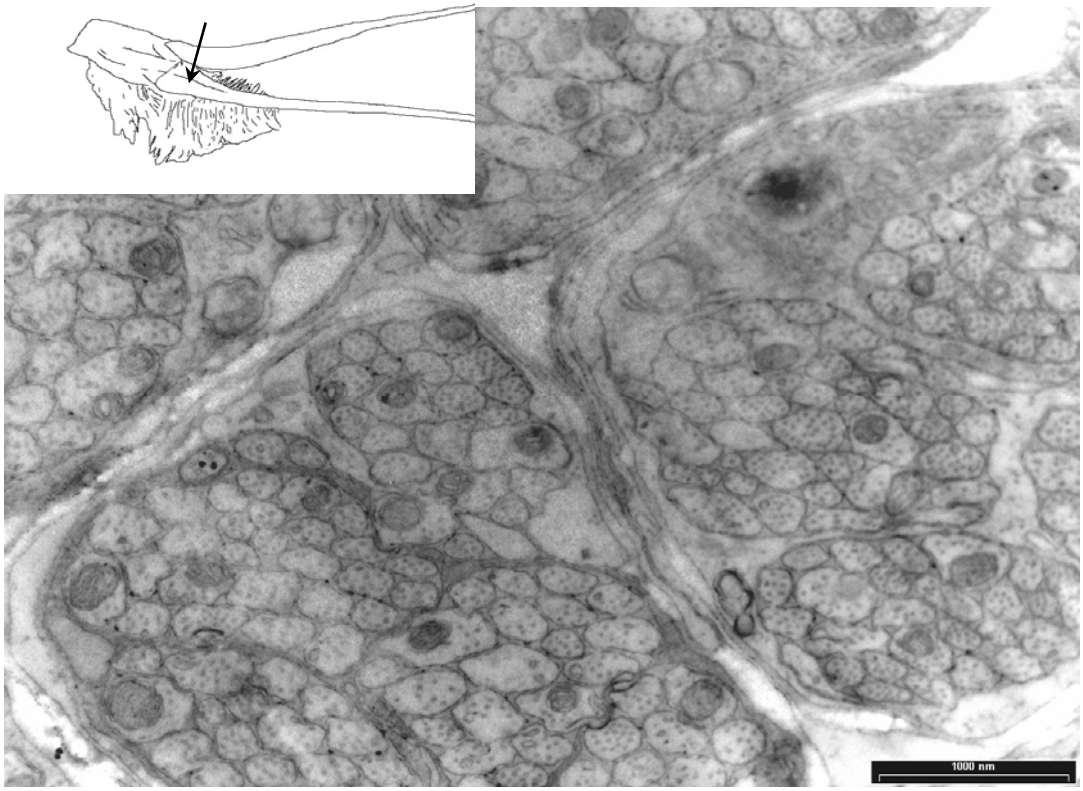


Figure 1.14. Transmission electron microscopy (TEM) photograph (5,000x mag.) showing bundles of olfactory axons that are between 0.1-0.2 microns in diameter and tightly packed within sheaths. These axons arise from olfactory receptor neurons and each bundle represents axonal groups from individual sensilla.

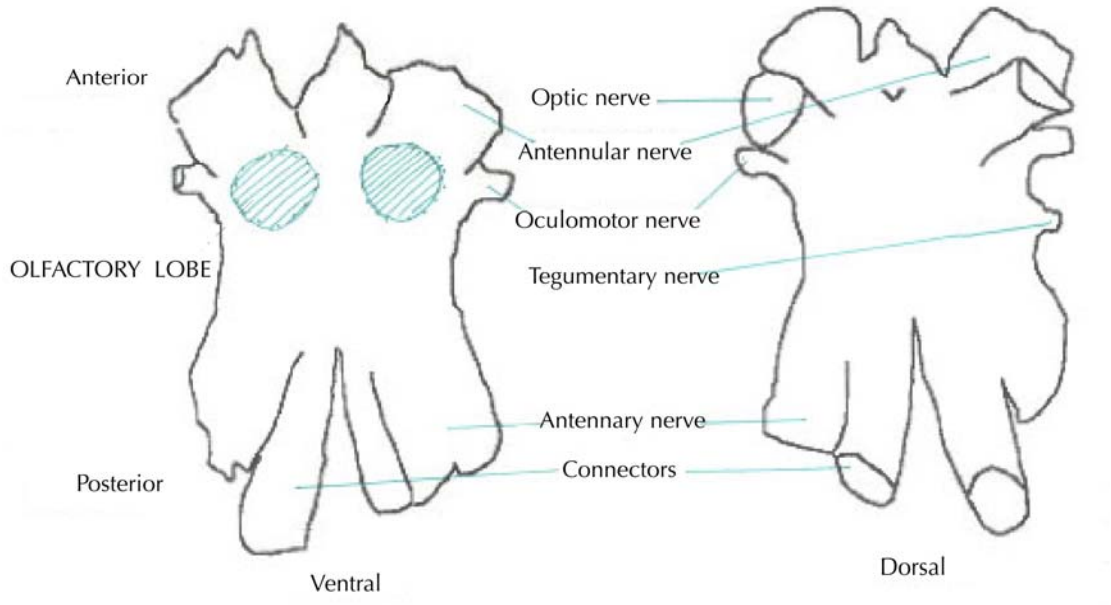


Figure 1.15. Ventral and dorsal view of a prawn brain showing the major nerves. The olfactory lobes (shown as shaded) receives the antennal nerve inputs and its volume represents a major proportion of the brain.

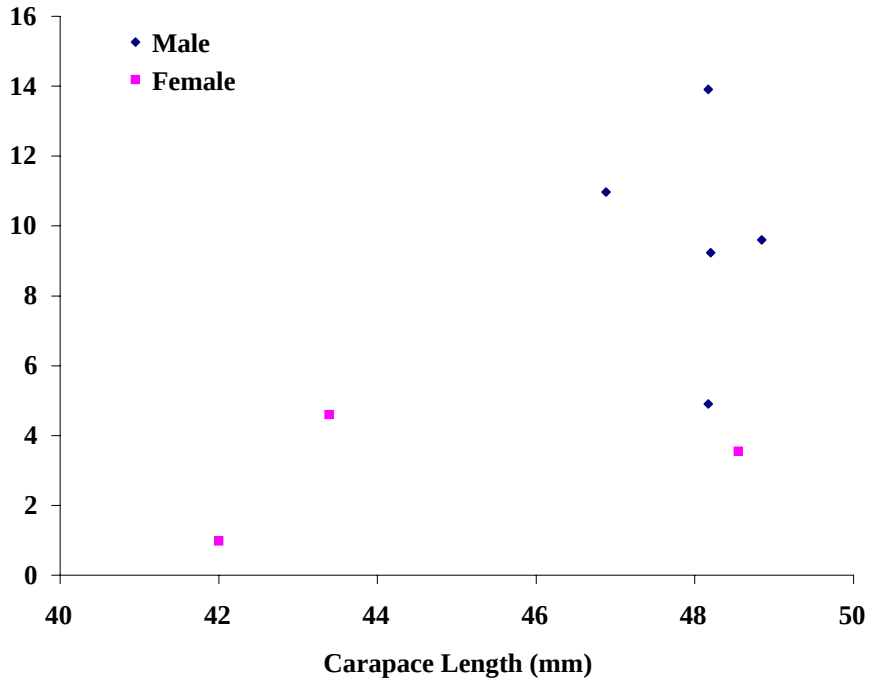


Figure 1.16. Comparison of volume of olfactory lobe in brain of male and female *Penaeus monodon*.

ELECTROPHYSIOLOGY OF CHEMORECEPTION

It was originally intended to maintain penaeid prawns at the University of New South Wales (UNSW) for neurophysiological experiments in an attempt to develop a neurological bioassay to identify pheromone candidates extracted from seawater. This approach would be used as an independent demonstration of the identification of pheromone candidates isolated from the solid phase matrix chromatography system and would supplement the whole animal bioassay approach. However, after transport of some adult penaeid prawns from AIMS to holding facilities at UNSW it was deemed unsuitable as it led to excessive stress and appeared to induce physiological changes to the extent that the prawns were not in a suitable condition for further study. To address this problem Professor Sandeman developed a portable neurophysiological laboratory that could readily be transported to AIMS. It was demonstrated that it was possible to set-up, and have operational, a portable neurophysiological laboratory so that prawns could be used for experiments directly from their holding tanks at AIMS without concerns over the physiological stress inherent in shipping animals to Sydney.

During the visit to AIMS and subsequently at UNSW it was established that extracellular recording from the axons within the isolated, but living, antennule nerve did not reveal biopotentials that reflected the responses of the chemoreceptor sensilla to any of the test 'positive control' substances. Several electrode configurations were attempted in which the summed activity of the receptors would have appeared as an "electroantennogram" of the kind routinely recorded from insects to detect candidate pheromone chemicals (Cosse et al. 1995). However, despite substantial efforts changes in action potential due to anything other than mecho-receptors could not be detected (Fig. 1.17).

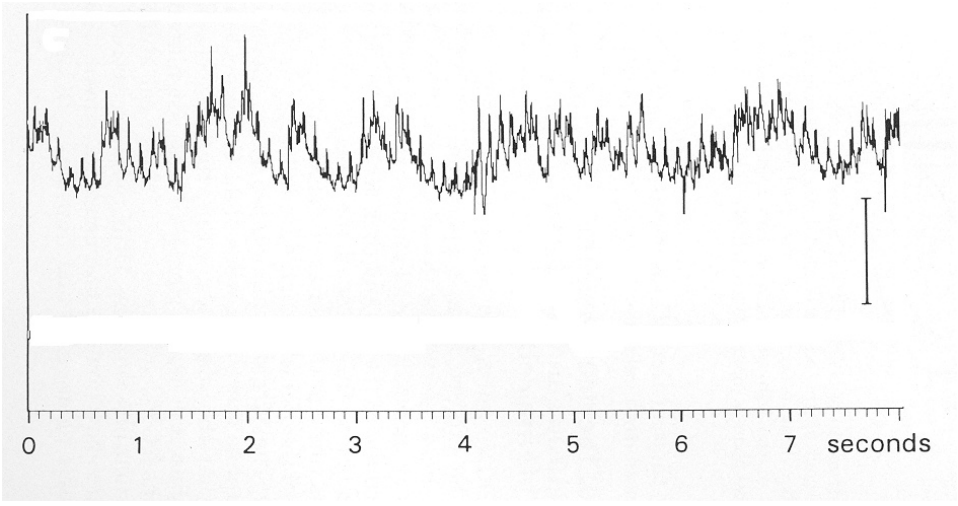


Figure 1.17. Electrophysiological recording from the antennule of a male prawn. No discernible changes in activity were noted upon exposure to known stimulatory attractants. The scale bar on the right equals 10 mV.

PHEROMONE SOURCE - ROSETTE GLANDS

Sections through the base of the antennae of prawns were examined by light microscopy for the presence of characteristically shaped glands indicative of rosette glands. Rosette-like glands were found and occupied the same area as that reported in lobsters (Figs. 1.18 and 1.19). If the rosette glands are the ones responsible for producing a pheromone attractive to the males these observations support the finding of more olfactory receptors in males to detect female pheromones.

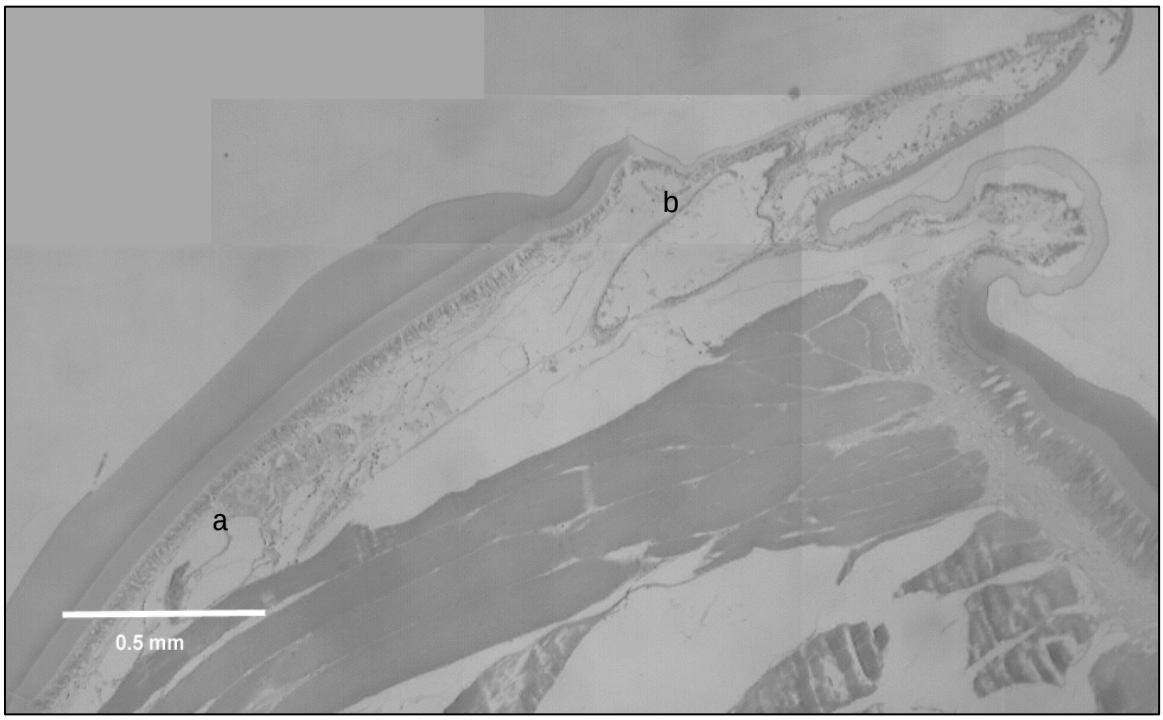


Figure 1.18. Rosette glands (a) present close to the uropore exit (b) in a female *Penaeus monodon*.

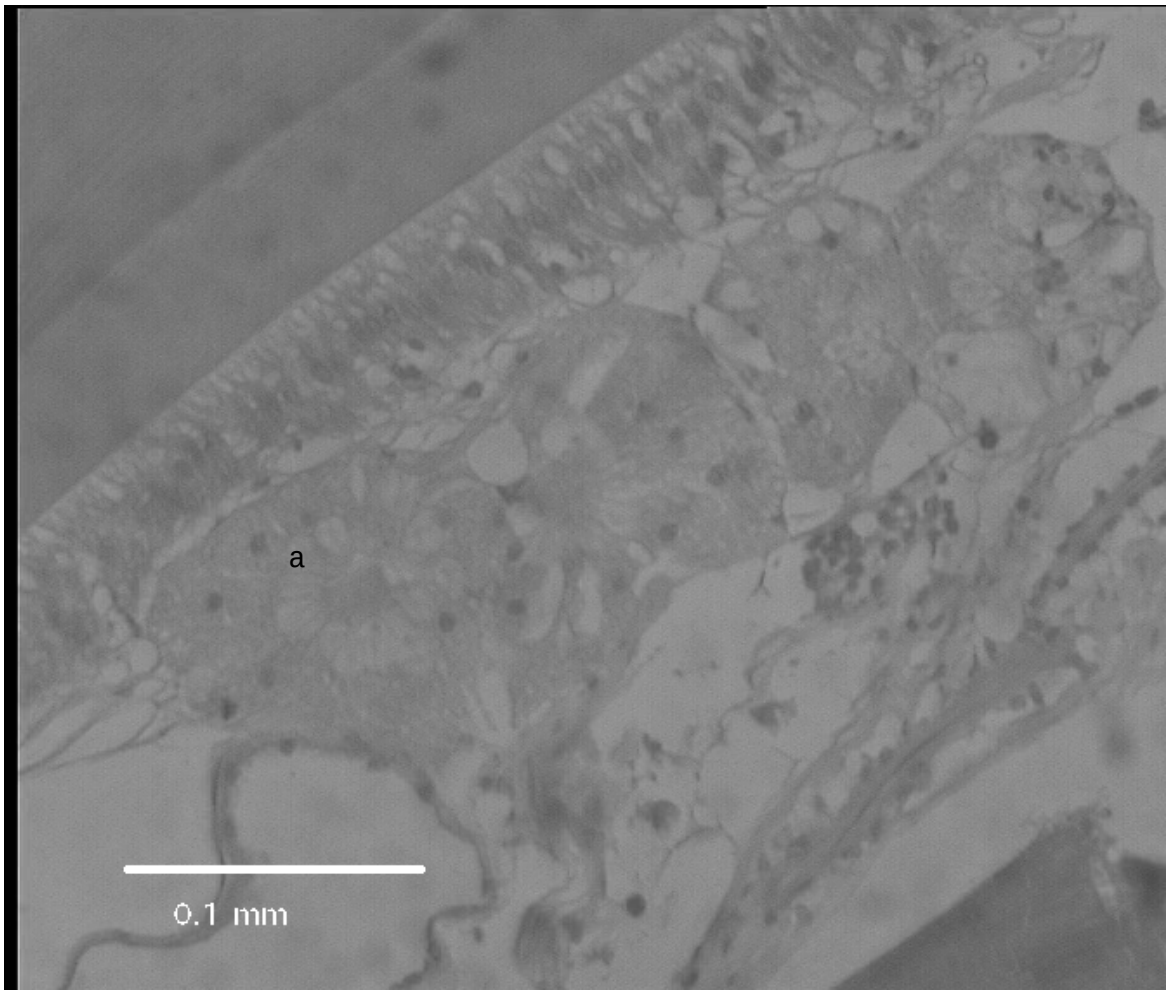


Figure 1.19. Rosette glands (a) in a female *Penaeus monodon*. The rosette gland cells have the characteristics of secretory cells. Tubules were observed from clusters of cells and are believed to empty into the urinary duct.

WHOLE ANIMAL BIOASSAY – ODOR TRACKING

Prawns could be held in the aquarium system developed for behavioural observations for weeks and appeared to readily adapt to this captive environment. Green tiger prawns buried themselves during the hours of daylight and only emerged after darkness.

There was a marked difference in behaviour in the prawns without and with a chemo-attractant. In the absence of a point source of a chemo-attractant the movements of the prawns were non-directional (Fig. 1.21). However, it also produced the first evidence that movement was not random in the tank. The prawns preferred to follow around the edges of the tank and rarely ventured into the main arena. The prawns moved in one direction until they made physical contact with their antennae with the side of the tank. At that point they turned either right or left and continued to follow the sides of the tank. This behavior, in effect, reduced

the area of the tank actually utilised by controls to a fraction of the total available. When a chemo-attractant odor flowed from one of the four channels (Fig. 1.21), in this case from channel 2 (second from the top of the figure) there was a distinct increase in odor tracking with prawns transversing the centrally areas of the tank in addition to control behavior of simply following the edges of the tank. The results demonstrated that prawns are capable of efficient odor tracking and locating the source of the odor of a food chemo-attractant.

Male prawns exhibited intense locomotive activity and extensive search behaviour in the arena bioassay tank when exposed to a feeding chemo-attractant. In contrast, searching activity of males did not vary significantly in the presence or absence of chemo-attractants emitting from a non-moulting or moulting female when held in the holding tank that overflowed into channel 2 (Figs.1. 22 and 1.23). Activity of male prawns also did not vary significantly (Figs. 1.24 and 1.25). If odor did form an organised plume so the prawn could not track it to its origin, a change in searching intensity might indicate the presence of a pheromone. The motion analysis data were therefore analyzed with respect to the time a prawn spent in any one sector of the tank (see Fig. 1.20). Specifically the presence or absence of a prawn in 9 sectors of the tank was examined; one of the 4 channels, one of 4 peripheries to the channels and the main arena itself (Fig. 1.26). As discussed above channels 1 and 4 are not of value in determining chemo-attraction as prawns usually tracked the sides of the tank. There was no indication that males could odor track a putative pheromone released by a moulting female nor any evidence that males searched a greater area of the entire tank.

As described in the methods section, motion tracking analysis of multi-prawns in one tank could not be automated by computer algorithms and all video analysis had to be analyzed frame by frame. Motion tracking analysis over approximately 1 hour did not reveal any odor tracking by male prawns as described above. In order to ensure that a bout of odor tracking was not being missed by only analyzing a short period of video footage another set of experiments examined for odor tracking behavior of males in the presence or absence of a moulting female was undertaken but this time by analyzing 3 hours of behavior before and after a female moulted (Figs. 1.27 and 1.28). Activity patterns were also examined over the same period (Figs. 1.29 and 1.30). No change in odor tracking or activity was observed in the presence or absence of a moulting female (Fig. 1.31 and Table 1.5).

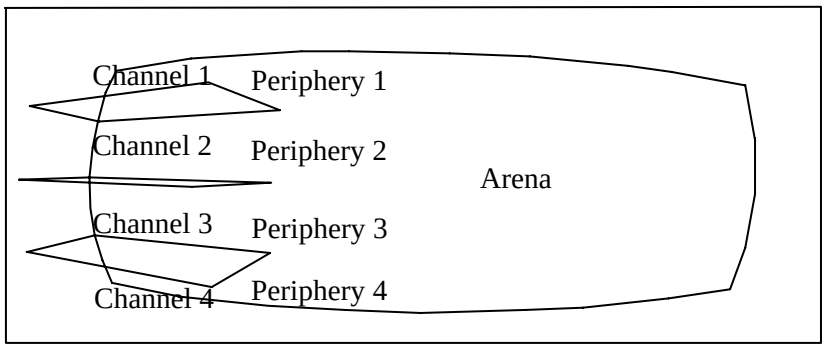


Figure 1.20. Diagrammatic representation of the 'olfactometer' tank subdivisions for computer motion analysis. The tank was divided into sectors, including the 4 channels, 4 periphery areas in front of the respective channel water discharge and the main arena.

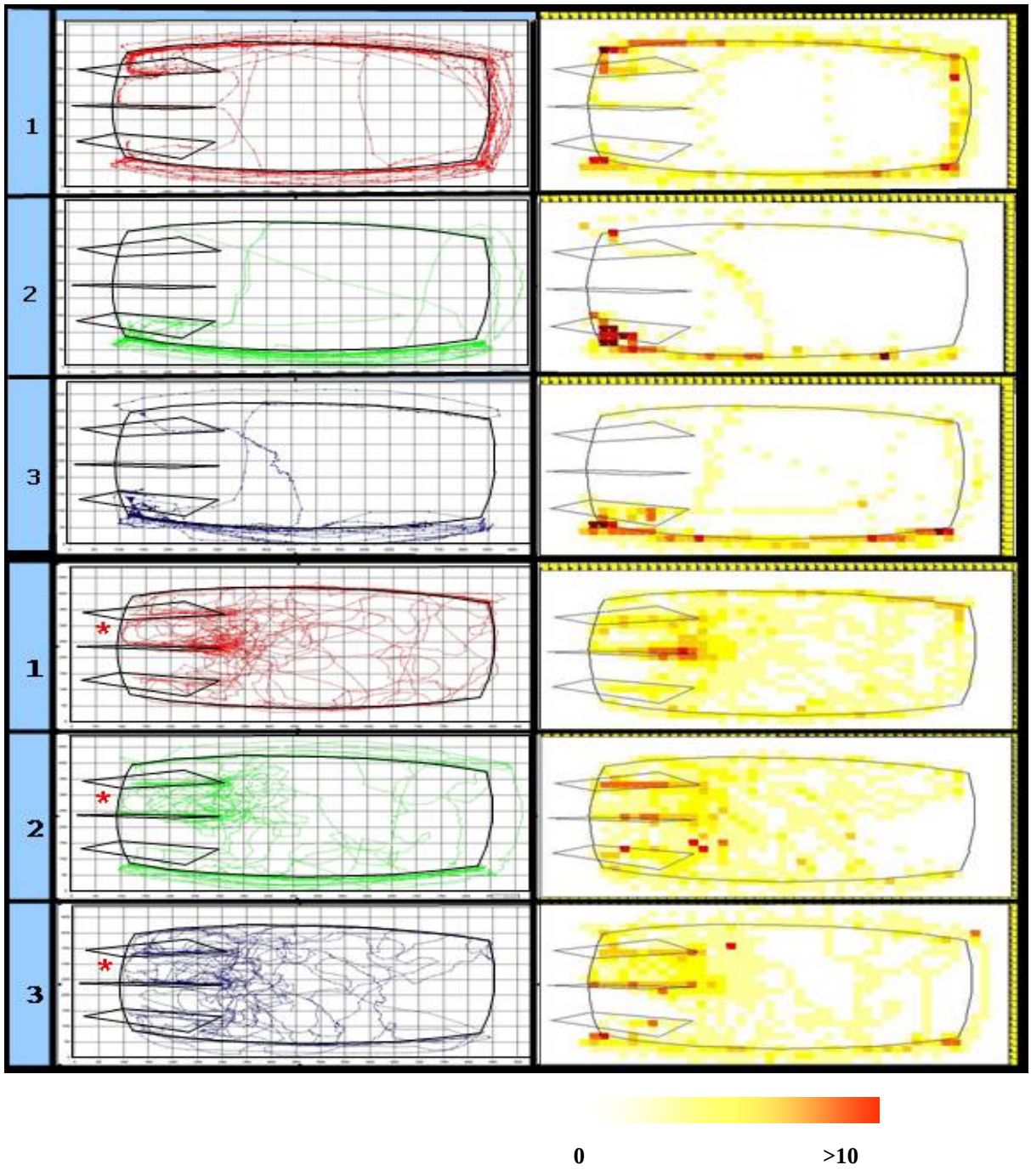


Figure 1.21. Motion tracking analysis of three male *P. semisulcatus* prawns in 'olfactometer' tank without chemo-attractant stimulus (upper 3 boxes on left hand side) and 'hot spot' locations (upper 3 boxes on right hand side) compared to a feeding chemo-attractant stimulus – in Channel 2 noted with * - (lower 3 boxes on left hand side) and 'hot spot' locations (lower 3 boxes on right hand side) where prawns spent their time during video recording, from 0 to >100 seconds total resident time over a 38 minute period.

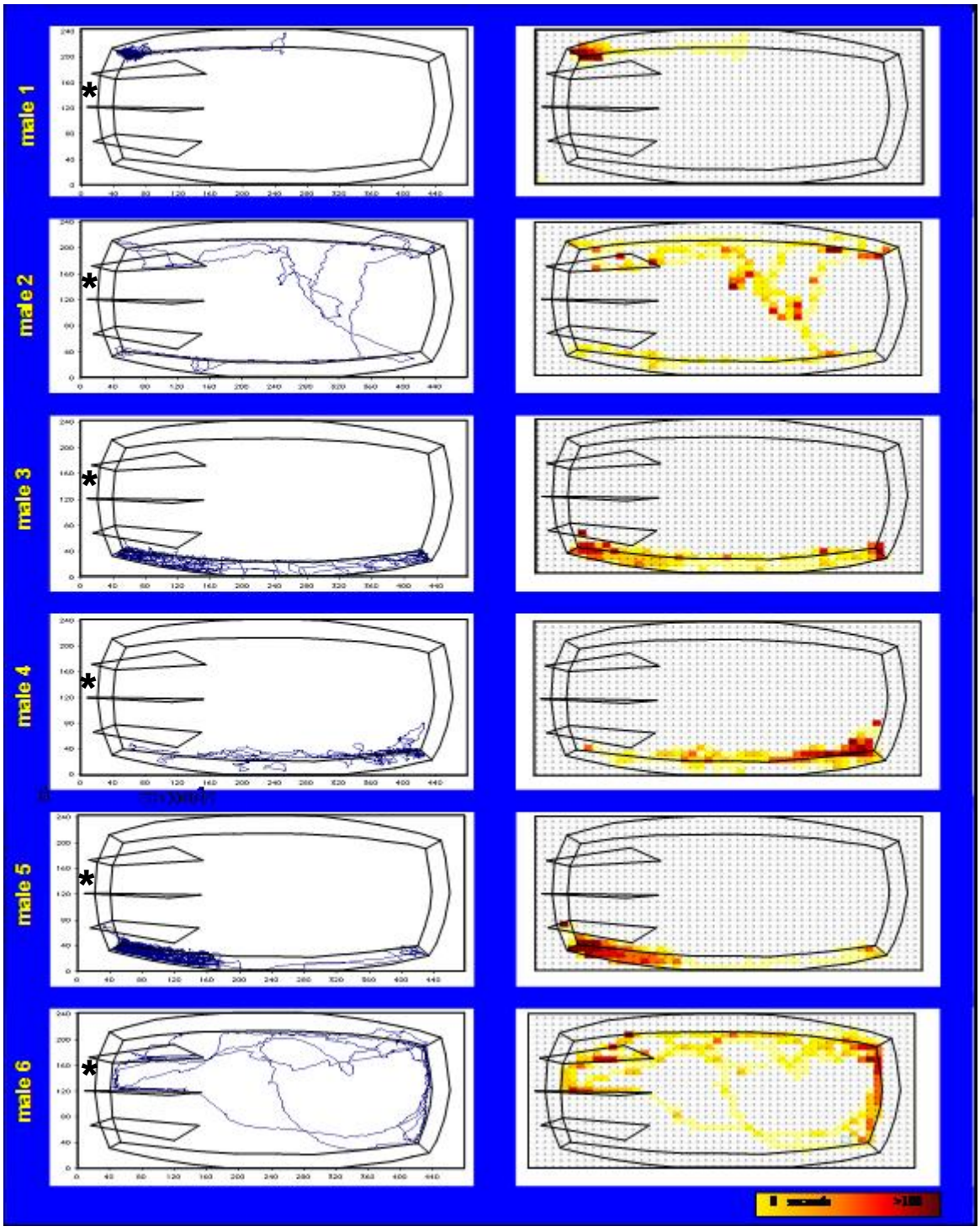


Figure 1.22. Motion tracking analysis of 6 male *P. semisulcatus* in 'olfactometer' tank exposed to overflow water through Channel 2 (shown marked with *) from non-moulting female. Analysis of 3,000 frames at 1 frame/sec over 50 minutes. 'Hot spot' locations from 0 to >100 seconds total resident time over recording period shown in right column.

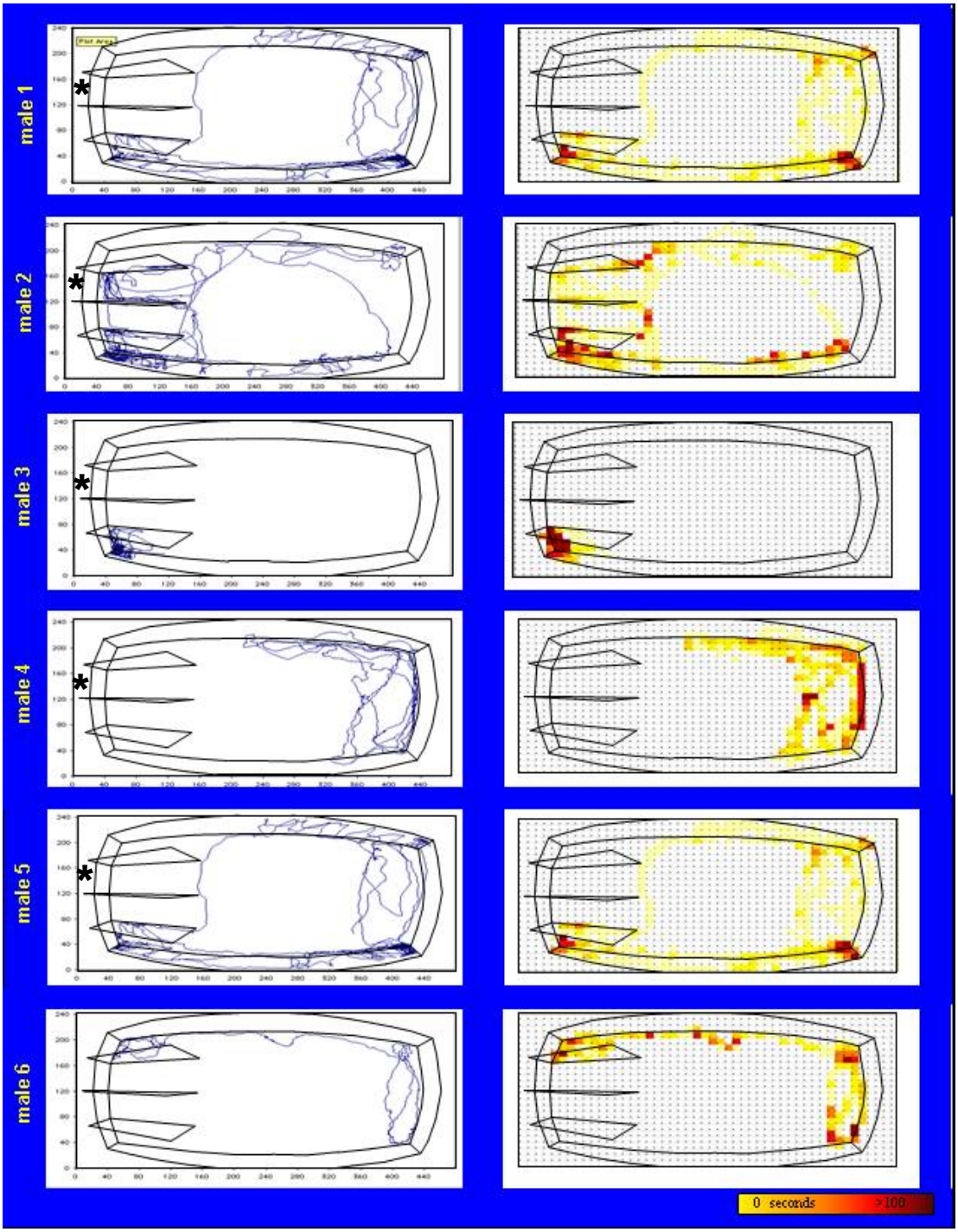


Figure 1.23. Motion tracking analysis of 6 male *P. semisulcatus* in 'olfactometer' tank exposed to overflow water through Channel 2 (shown marked with *) from moulting female. Analysis of 3,000 frames at 1 frame/sec over 50 minutes. 'Hot spot' locations from 0 to >100 seconds total resident time over recording period shown in right column.

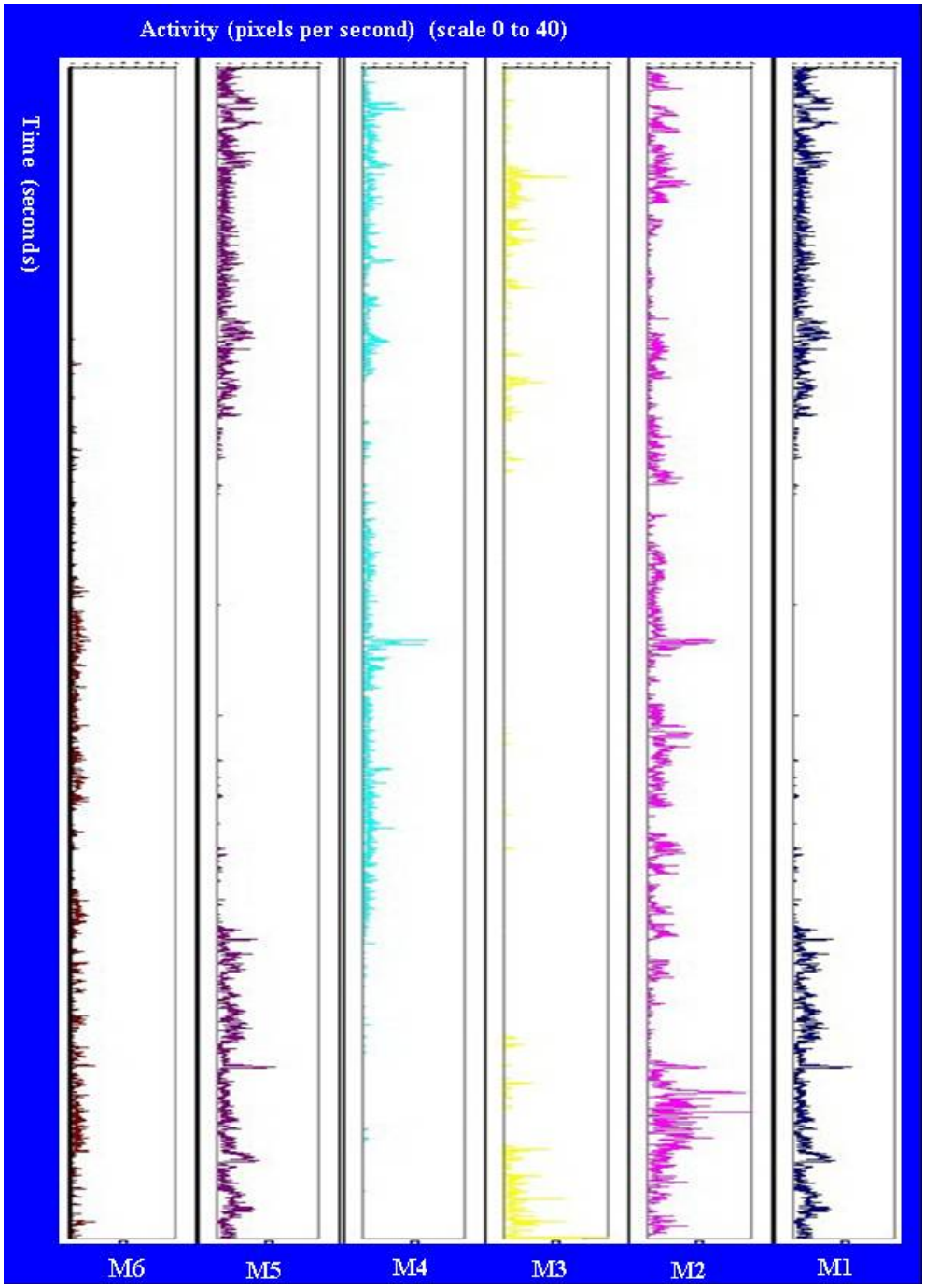


Figure 1.24. Activity plot of 6 males in olfactometer tank exposed to over flow water from a non-moulting female (cf Fig. 1.22). Data expressed in pixels moved per second as one measure of odor bioassay.

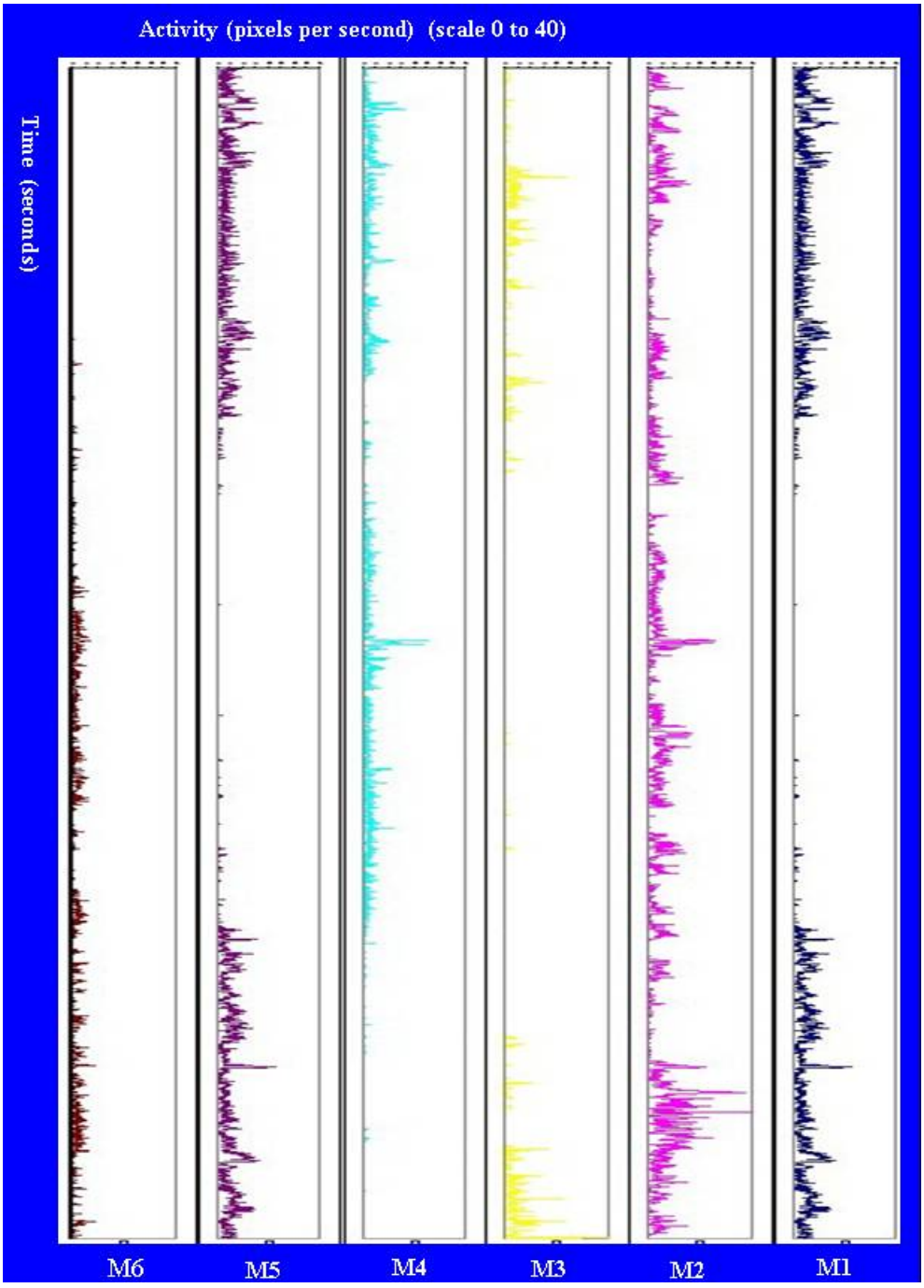


Figure 1.25. Activity plot of 6 males in olfactometer tank exposed to over flow water from a moulting female (cf Fig. 1.22). Data expressed in pixels moved per second as one measure of odor bioassay.

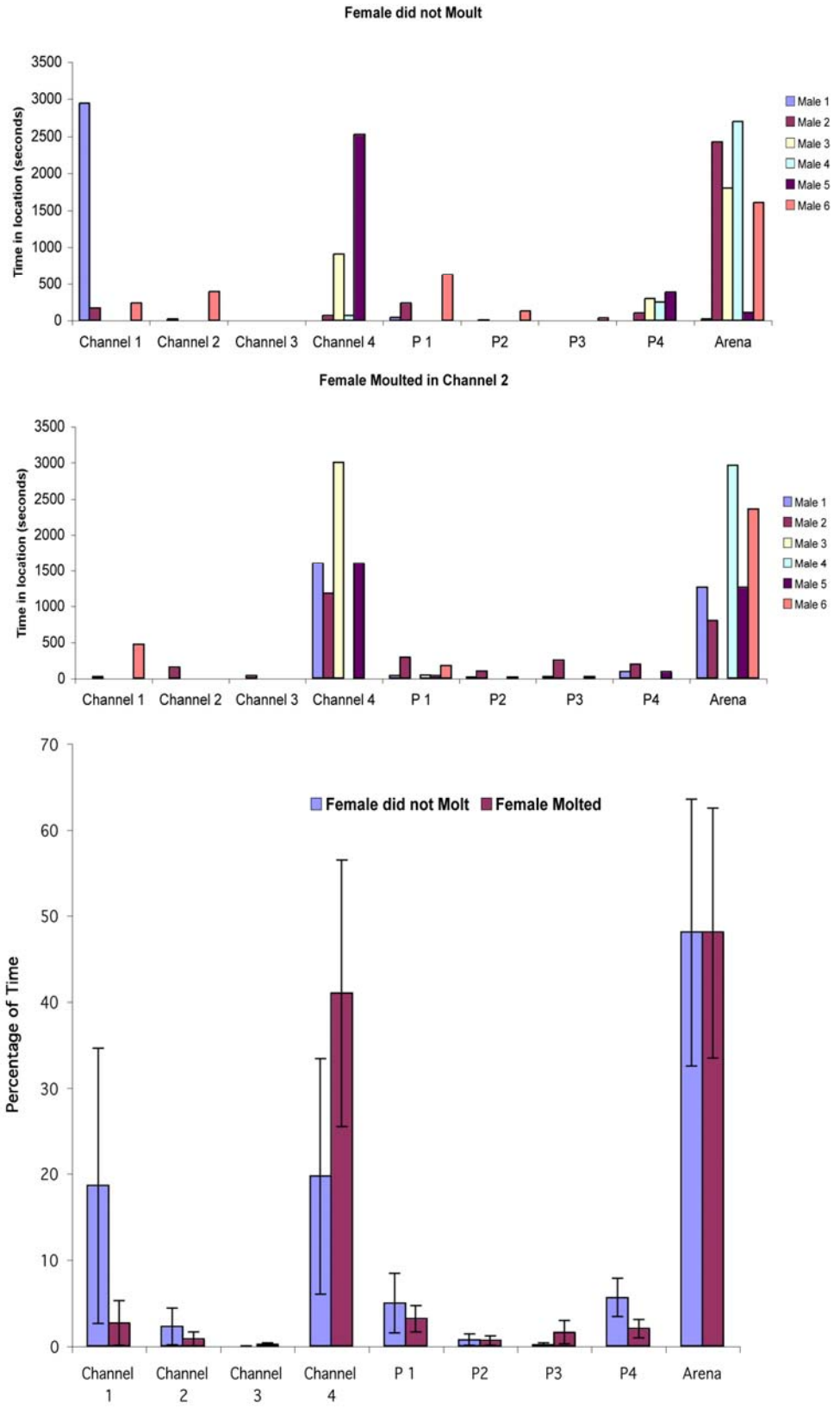


Figure 1.26. Histogram of time individual prawns spent in nine specific areas of the 'olfactometer' tank.

Figure 1.27. Motion tracking analysis of 5 male *P. semisulcatus* in 'olfactometer' tank exposed to overflow water through Channel 2 from non-moulting female. Analysis of motion tracking over 3 hours in 1 hour intervals (3,600 frames at 1 frame/sec over 1 hour). Far left column shows total motion tracking over 3 hours whereas subsequent columns to the right are sequential 1 hour plots. 'Hot spot' locations from 0 to >100 seconds total resident time over recording period shown in below each respective motion tracking analysis box.

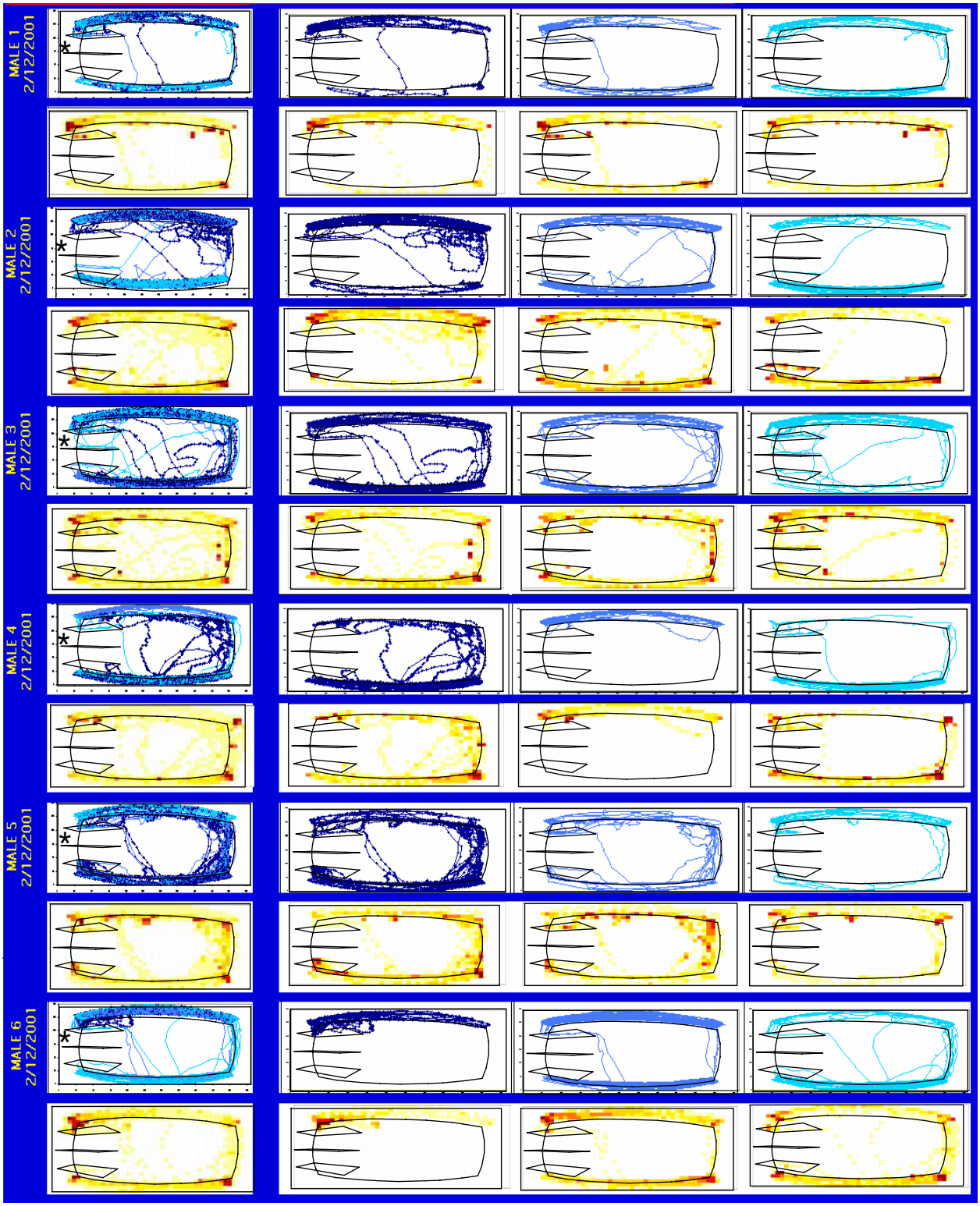


Figure 1.28. Motion tracking analysis of 6 male *P. semisulcatus* in 'olfactometer' tank exposed to overflow water through Channel 2 from moulting female. Analysis of motion tracking over 3 hours in 1 hour intervals (3,600 frames at 1 frame/sec over 1 hour). Far left column shows total motion tracking over 3 hours whereas subsequent columns to the right are sequential 1 hour plots. 'Hot spot' locations from 0 to >100 seconds total resident time over recording period shown in below each respective motion tracking analysis box.

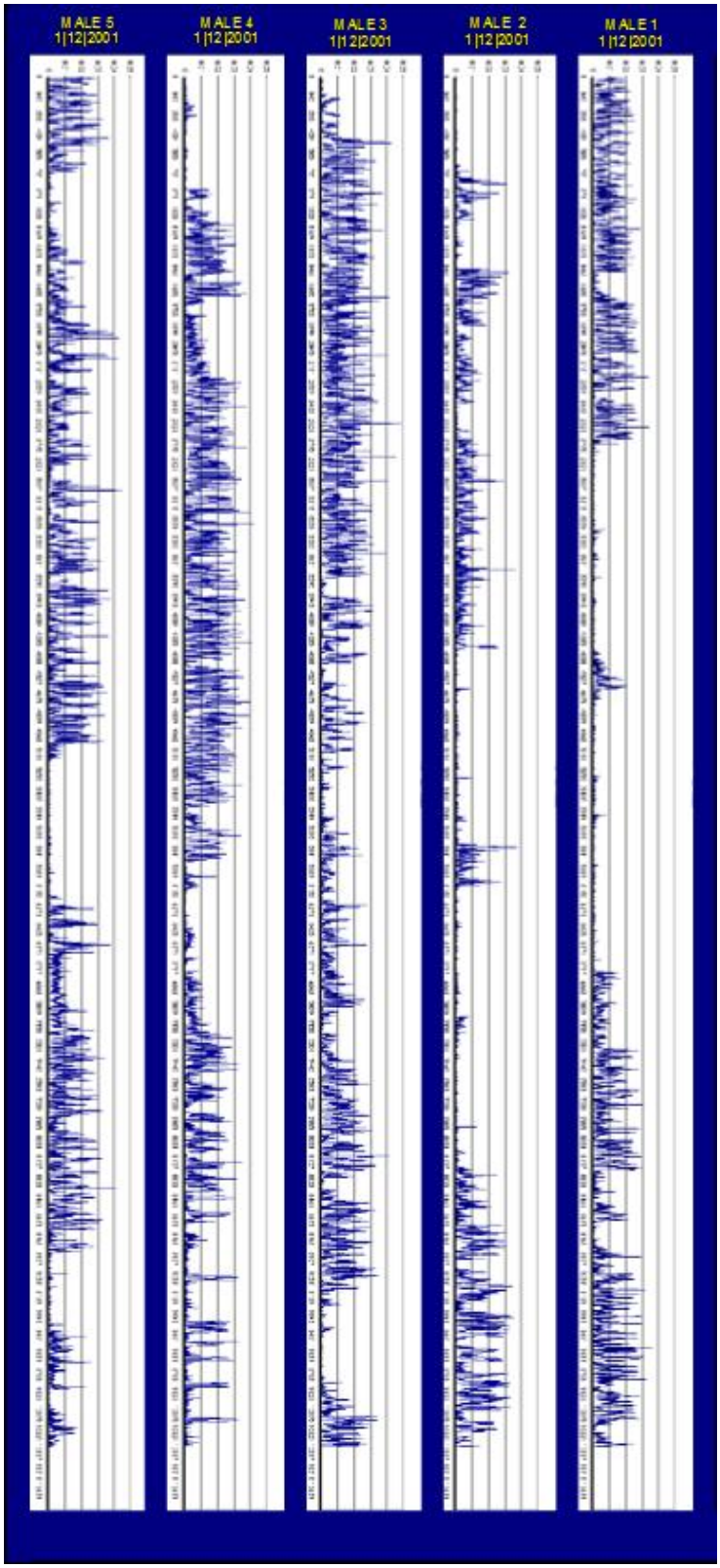


Figure 1.29. Activity plot of 5 males in olfactometer tank exposed to over flow water from a non-moulting female (cf Fig. 1.22). Data expressed in pixels moved per second as one measure of odor bioassay. If odor did form an organised plume so the prawn could not track it to its origin, a change in searching intensity might indicate the presence of a pheromone.

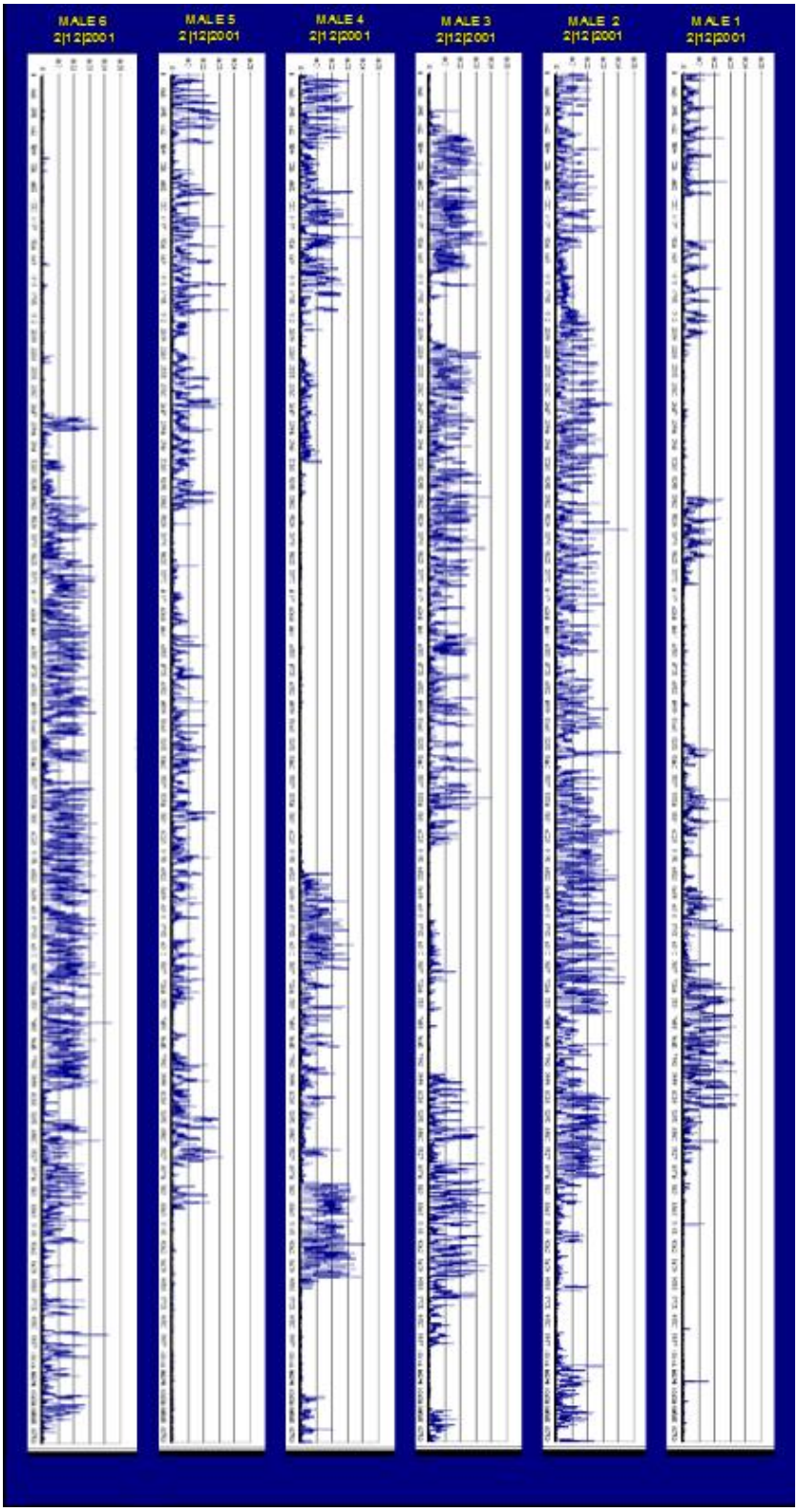


Figure 1.30. Activity plot of 6 males in olfactometer tank exposed to over flow water from a moulting female (cf Fig. 1.22). Data expressed in pixels moved per second as one measure of odor bioassay. If odor did form an organised plume so the prawn could not track it to its origin, a change in searching intensity might indicate the presence of a pheromone.

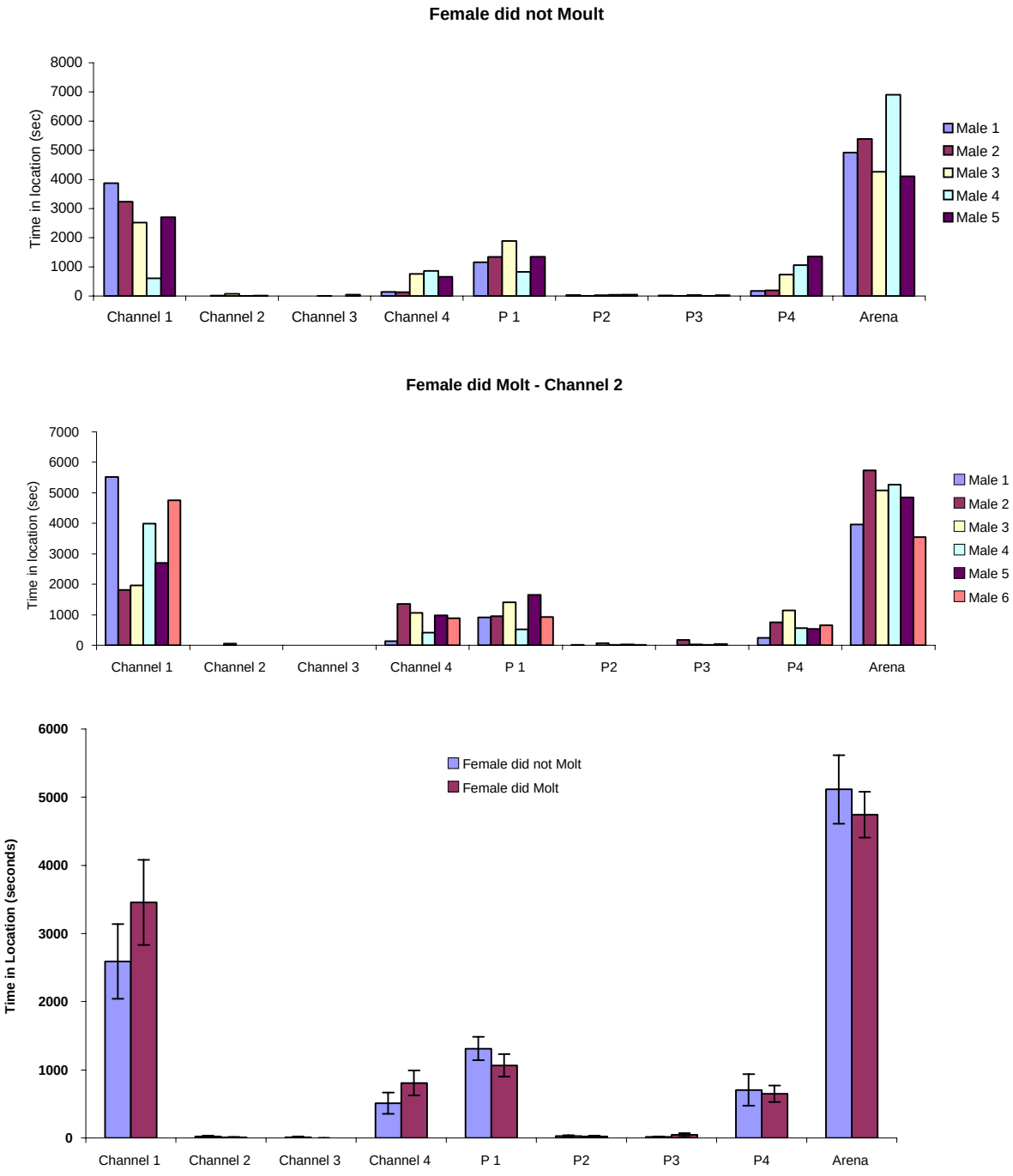


Figure 1.31. Time spent within specific sectors of the 'olfactometer' tank from 2100 to 2300 hours of 5 to 6 male prawns with females in divider 2 that did not moult (above) and a when female did moult in holding tank with overflow water in Channel 2 header (below). See Figure 1.20 for details.

Table 1.5. Tabulated time (seconds) spent within specific sectors of the 'olfactometer' tank from 2100 to 2300 hours of 5 to 6 male prawns with females in divider 2 that did not moult (NM) and when a female did moult (M) in holding tank with overflow water in Channel 2 header. See Figures 1.27 and 1.28 for details.

	C 1	C 2	C 3	C 4	P 1	P2	P3	P4	Arena	Total
FEMALE DID NOT MOULT										
M1 NM 2000 hrs	2030	0	0	0	718	0	0	0	852	3600
M1 NM 2100 hrs	734	0	0	21	55	26	18	64	2682	3600
M1 NM 2200 hrs	1108	0	0	118	385	7	5	111	1388	3122
M1 NM 2000-2300 hrs	3872	0	0	139	1158	33	23	175	4922	10322
M2 NM 2000 hrs	1772	0	0	0	1027	0	0	0	801	3600
M2 NM 2100 hrs	591	14	0	77	35	5	2	79	2797	3600
M2 NM 2200 hrs	875	0	0	57	281	2	2	118	1787	3122
M2 NM 2000-2300 hrs	3238	14	0	134	1343	7	4	197	5385	10322
M3 NM 2000 hrs	406	0	0	368	315	3	1	525	1982	3600
M3 NM 2100 hrs	992	76	10	374	496	24	31	191	1406	3600
M3 NM 2200 hrs	1127	0	0	19	1079	0	0	24	873	3122
M3 NM 2000-2300 hrs	2525	76	10	761	1890	27	32	740	4261	10322
M4 NM 2000 hrs	135	0	0	259	353	2	0	406	2445	3600
M4 NM 2100 hrs	0	7	0	389	325	38	6	435	2400	3600
M4 NM 2200 hrs	479	0	0	213	150	2	2	219	2057	3122
M4 NM 2000-2300 hrs	614	7	0	861	828	42	8	1060	6902	10322
M5 NM 2000 hrs	259	12	52	485	727	35	29	418	1583	3600
M5 NM 2100 hrs	2003	0	0	37	422	15	0	64	1059	3600
M5 NM 2200 hrs	443	0	0	139	200	2	2	871	1465	3122
M5 NM 2000-2300 hrs	2705	12	52	661	1349	52	31	1353	4107	10322
Average NM	2590	21.8	12.4	511.2	1313.6	32.2	19.6	705	5115.4	
SE	547	13.8	10.1	156.2	172.4	7.6	5.8	233.0	502.4	
FEMALE MOULTED										
M1 M 2000 hrs	2688	0	0	6	376	3	0	27	500	3600
M1 M 2100 hrs	2301	0	0	85	344	14	4	141	711	3600
M1 M 2200 hrs	535	0	0	45	199	0	0	68	2753	3600

M1 M 2000-2300 hrs	5524	0	0	136	919	17	4	236	3964	10800
M2 M 2000 hrs	995	0	0	138	434	4	0	45	1984	3600
M2 M 2100 hrs	483	0	0	385	366	0	53	362	1951	3600
M2 M 2200 hrs	341	0	1	840	154	1	127	339	1797	3600
M2 M 2000-2300 hrs	1819	0	1	1363	954	5	180	746	5732	10800
M3 M 2000 hrs	491	0	0	224	251	10	5	337	2282	3600
M3 M 2100 hrs	390	0	0	726	237	0	0	217	2030	3600
M3 M 2200 hrs	1080	50	0	107	922	60	20	589	772	3600
M3 M 2000-2300 hrs	1961	50	0	1057	1410	70	25	1143	5084	10800
M4 M 2000 hrs	580	0	0	139	164	10	17	252	2438	3600
M4 M 2100 hrs	3076	0	0	0	217	0	0	0	307	3600
M4 M 2200 hrs	333	0	0	276	148	9	1	314	2519	3600
M4 M 2000-2300 hrs	3989	0	0	415	529	19	18	566	5264	10800
M5 M 2000 hrs	113	0	0	539	338	7	7	353	2243	3600
M5 M 2100 hrs	245	0	0	350	772	18	31	155	2029	3600
M5 M 2200 hrs	2345	0	0	98	547	0	0	27	583	3600
M5 M 2000-2300 hrs	2703	0	0	987	1657	25	38	535	4855	10800
M6 M 2000 hrs	3149	0	0	0	341	0	0	0	110	3600
M6 M 2100 hrs	1071	0	0	146	474	9	4	311	1585	3600
M6 M 2200 hrs	535	0	0	741	112	3	2	350	1857	3600
M6 M 2000-2300 hrs	4755	0	0	887	927	12	6	661	3552	10800
Average M	3458	8.3	0.2	807.5	1066	24.7	45.2	647.8	4741.8	
SE	625	8.3	0.2	183.8	164.3	9.5	27.4	121.7	336.8	

Video footage of male behavior when a pre-moulting female is actually in the main arena tank itself, demonstrates a dramatic behavioural response in the males compared to other non-moulting females in the same tank (video file available but not shown) (Figs. 1.32, 1.33 and 1.34). The pattern of overall male activity was also increased in the presence of a moulting female (Figs. 1.35, 1.36 and 1.37). There is frequent transient contact of males with the female and eventually mating. Activity plotted as pixels covered (equivalent to distance) per second over time is shown in Figure 1.38. Total movement, or area covered, of a female was nearly three-fold higher on the night a female moulted compared when she did not moult. Males

increased their overall movement activity by up to seven-fold on the night a female in the arena moulted. It is unclear, however, how much of this behaviour was due to a released pheromone compared to a contact pheromone. Males exhibit frenzied tactile behavior once physical contact is made with the female. Such behavior is indicative of a contact pheromone.

Figure 1.32. Motion tracking analysis of 4 male and 2 female *P. semisulcatus* in 'olfactometer' tank approximately 1 hour before one of the females moulted in the tank.

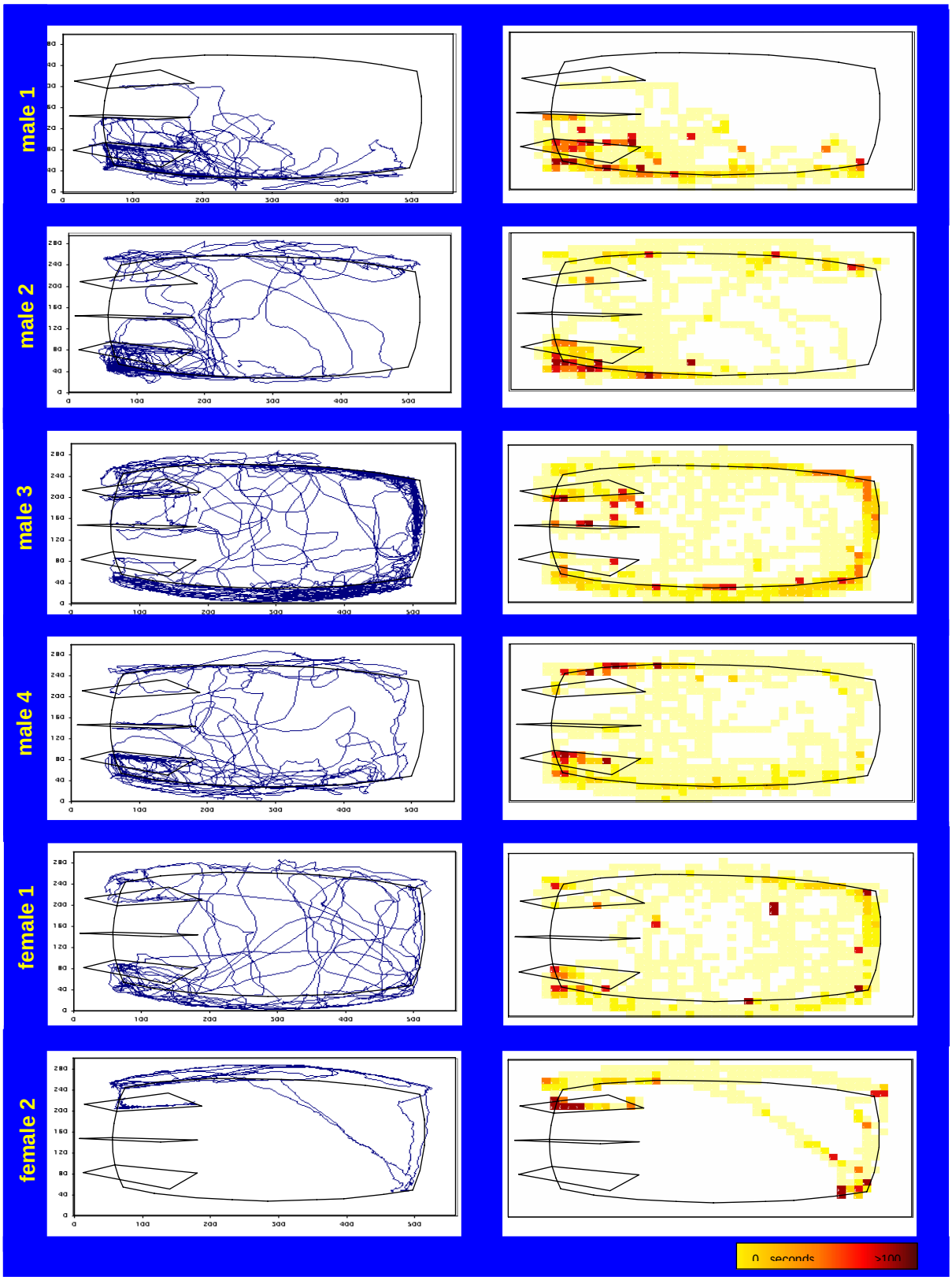


Figure 1.33. Motion tracking analysis of 4 male and 2 female *P. semisulcatus* in 'olfactometer' tank over 1 hour period when one of the females moulted in the tank.

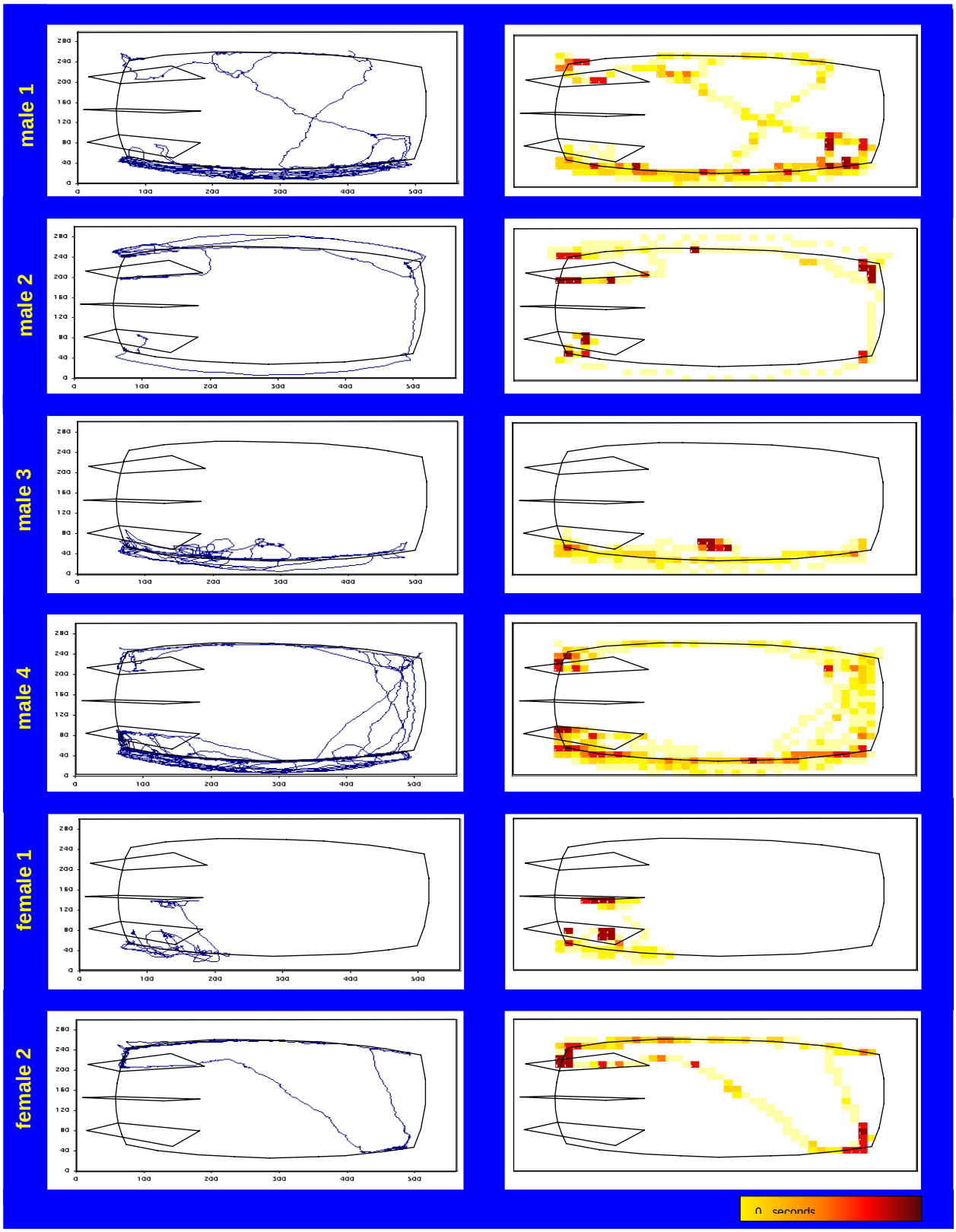


Figure 1.34. Motion tracking analysis of 4 male and 2 female *P. semisulcatus* in 'olfactometer' tank when no female moulted in the tank but at the same time of night as that shown in Fig. 1.33.

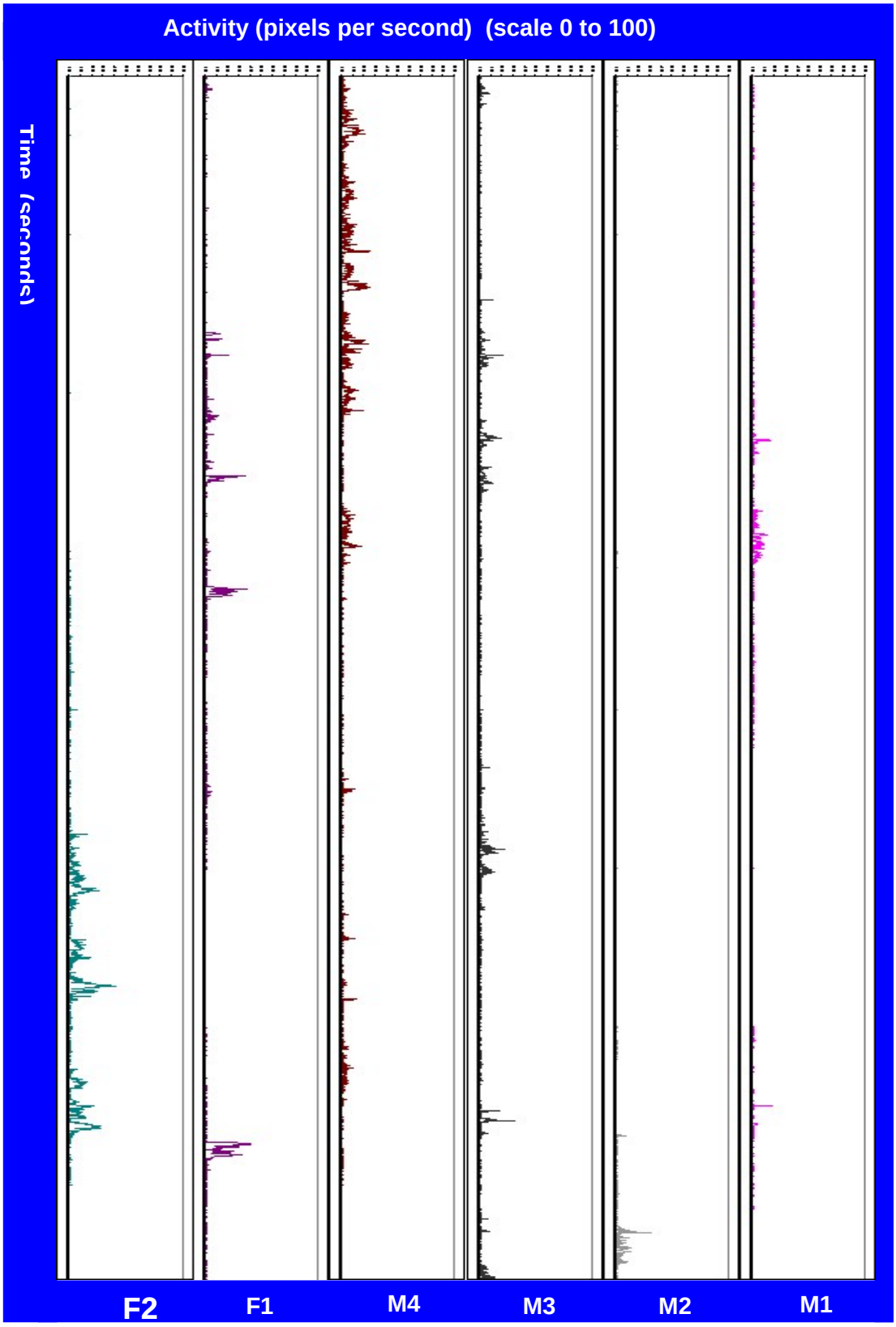


Figure 1.35. Activity analysis of 4 male and 2 female *P. semisulcatus* in 'olfactometer' tank approximately 1 hour before one of the females moulted in the tank.

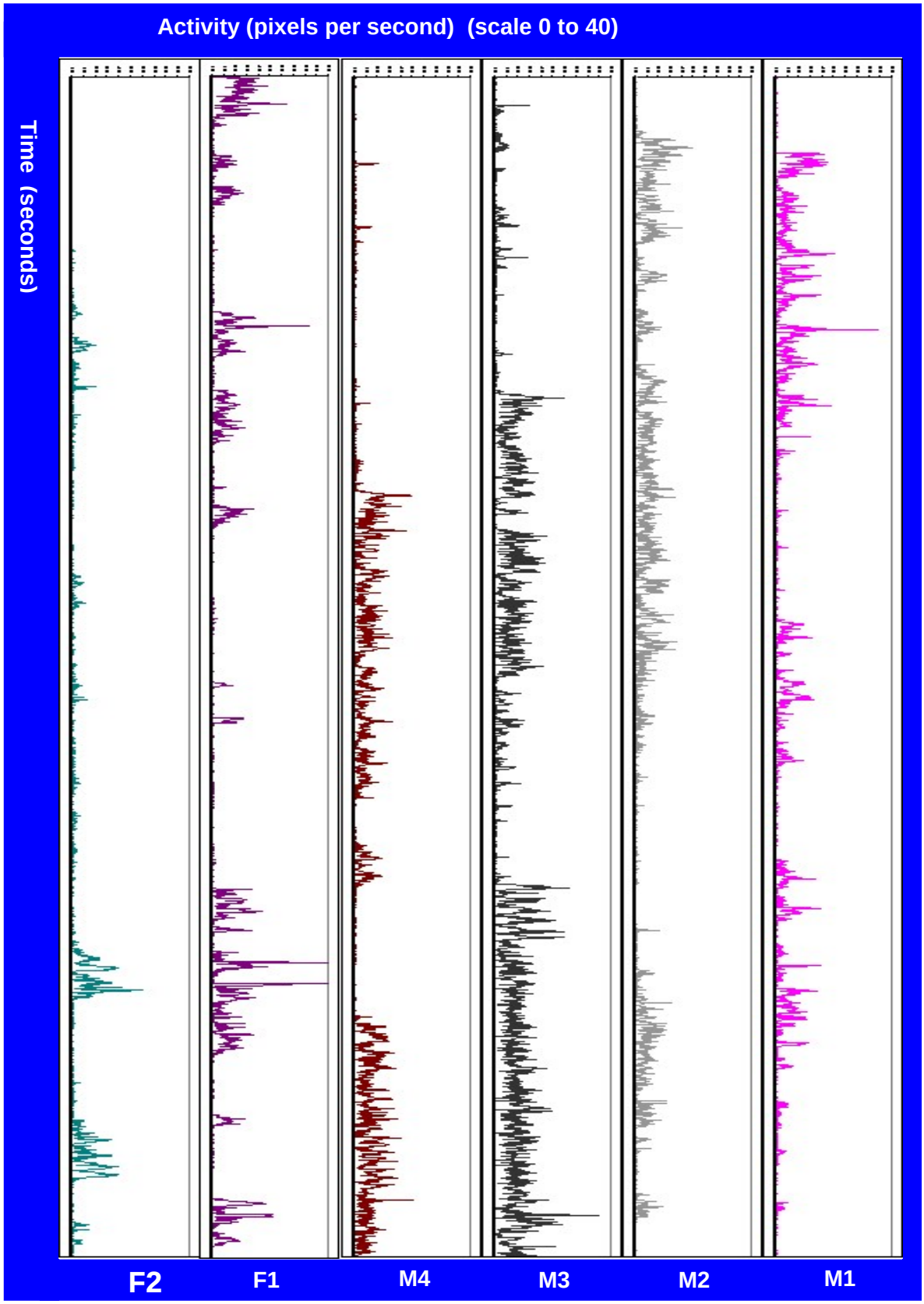


Figure 1.36. Activity analysis of 4 male and 2 female *P. semisulcatus* in 'olfactometer' tank over 1 hour period when one of the females moulted in the tank.

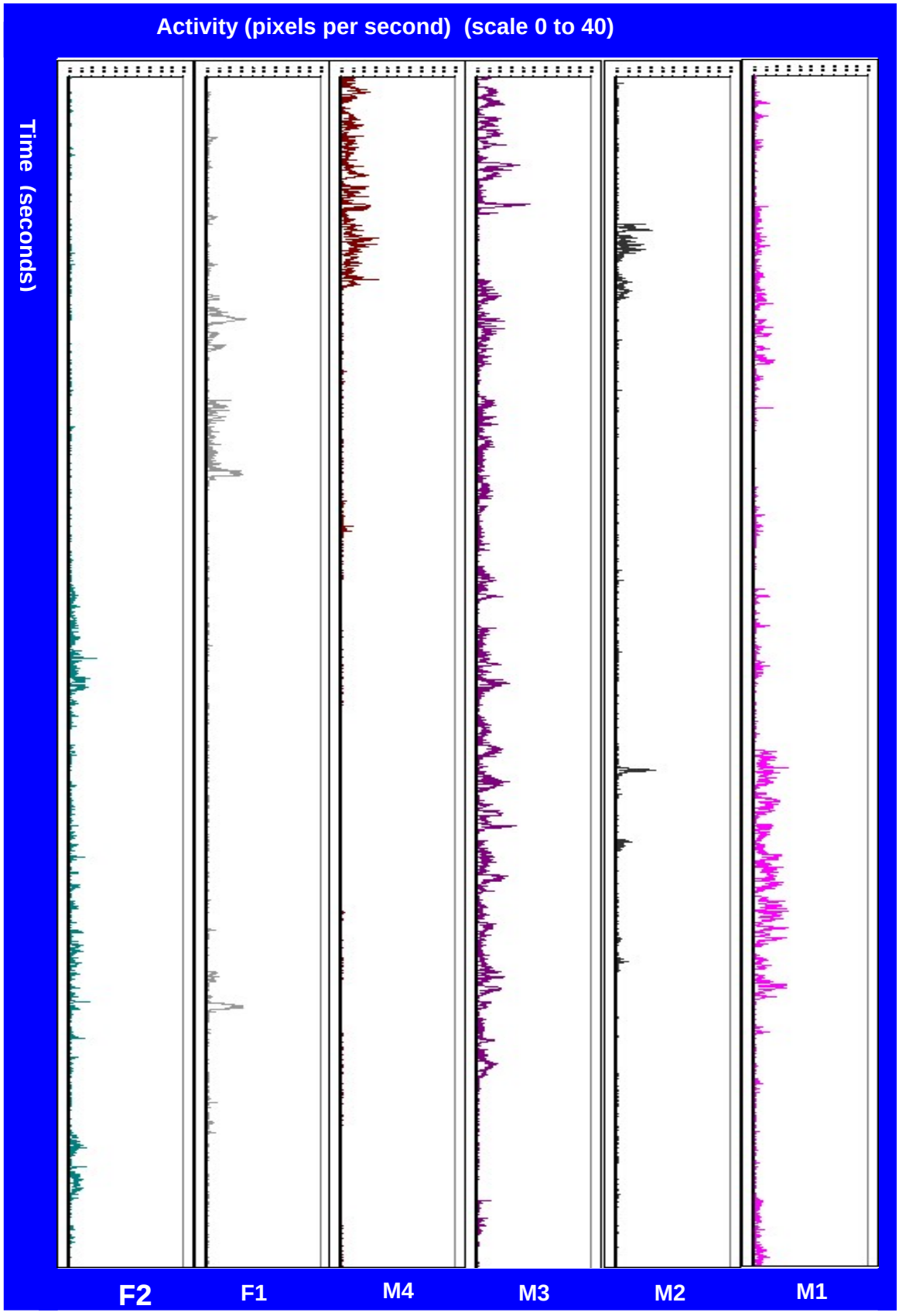


Figure 1.37. Activity analysis of 4 male and 2 female *P. semisulcatus* in 'olfactometer' tank when no female moulted in the tank but at the same time of night as that shown in Fig. 1.33.

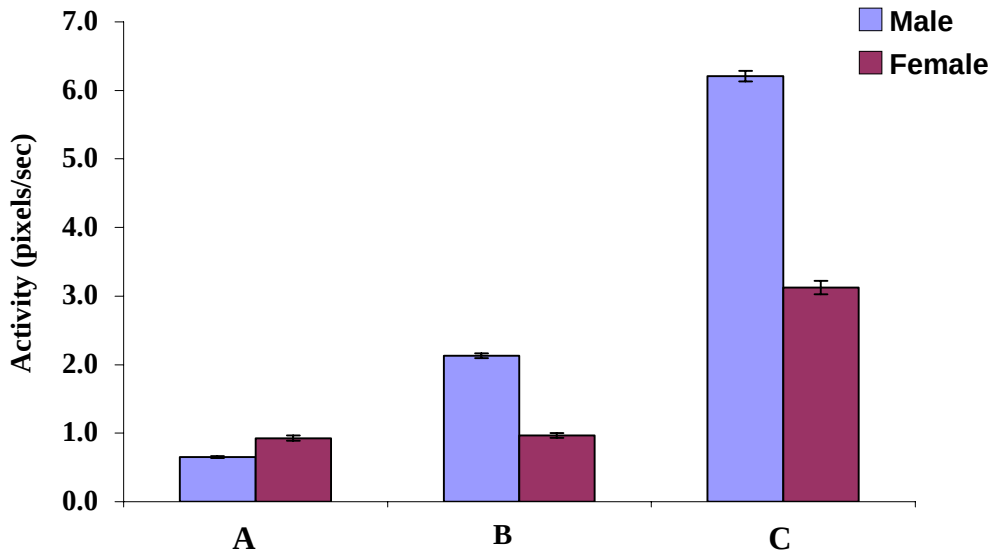


Figure 1.38. Histogram of activity of male and female prawns in the tank at specific times during the night. Activity of prawns 1 hour before a female moulted in the tank (A). Activity of prawns on a when no moult occurred at night (B) but at the same time of night of that in (C) which is the activity of prawns during the hour that a female moulted.

Discussion

CHEMORECEPTORS

The number of aesthetascs found on each antennule in *P. monodon* was much higher than that reported for Reptantian decapods whose size at sexual maturity is much greater than *P. monodon* (Beltz et al. 2002). In other decapods, the addition as well as loss of antennal segments and their aesthetascs has been reported to take place in the proximal and distal part of the lateral flagellum respectively (Steullet et al. 2000). It was also reported that new aesthetascs were added to rows on proximal segments of the flagellum following a moult and that following an additional moult the segment bears the full compliment of aesthetascs. The positioning of the aesthetasc rows on *P. monodon* differs from that of palinurid lobsters and brachyuran crabs where rows are found along the distal portion of the antennule (Beltz et al. 2002). In penaeids, aesthetascs appear to arise from the base of the lateral flagellum and terminate as the flagellum restricts midway. This variation in development may explain why additional aesthetascs are added to existing aesthetasc rows in penaeids as well as additional segments through division of the proximal region of the flagellum.

By measuring the length of aesthetasc rows over a range of body sizes in *P. monodon* we observed a net increase in the length of rows and number of aesthetascs per row as body size increased. This suggests additional aesthetascs are added to rows through the whole flagellum as the animal grows, as has been reported for the freshwater crayfish *Cherax destructor* (Sandeman and Sandeman 1996) and palinurid lobsters (Stuellet et al. 2000). This infers that the full complement of aesthetasc sensilla per flagellum segment will change with development. The negative allometry described in aesthetasc measurements from female shrimp suggests a full development of the aesthetasc antennules over examined body size. In *P. monodon* the addition of aesthetascs per flagellum segment was much greater than that seen with other decapods studied (Sandeman and Sandeman 1996, Steullet et al. 2000).

Comparison of the left and right lateral flagella showed consistent symmetry in the number of aesthetascs per flagellum. The variability in the length of the rows may have resulted from damage and regeneration of aesthetascs. For example, a dramatic increase in lobster aesthetasc numbers occurs following a moult in antennules in which the aesthetasc rows has been damaged (Steullet et al. 2000). The aesthetasc symmetry found in antennules would optimise the collection of directional information from chemical stimulus (Hildebrand and Shepard 1997). Lobsters that are either unilaterally antennule ablated or have their aesthetascs removed lose their ability to extract directional information from a chemical stimulus field (Devine and Atema 1982).

Sexual dimorphism in aesthetasc counts became more pronounced as the animals increased in body size (see Fig. 1.11). For example, an adult male with a carapace length of 43mm has 3205 sensilla whereas a female with a carapace length of 47 mm has 1201 sensilla. If the aesthetascs are involved in the detection of pheromones, it would appear that the males are better equipped than the females. Additional consequences of this are thought to be an increase in olfactory sensitivity associated with more olfactory receptor neurons (Beltz et al. 2002). Male *P. monodon* attain sexual maturity (develop mature spermatozoa) when they grow to greater than 37 mm in carapace length (Motoh 1981). Both male and females less than 30mm carapace length have comparable numbers of aesthetascs as shown by the intersection of the two regression slopes in this study. Male *P. monodon* appear to acquire additional aesthetascs just prior to sexual maturity represented by a divergence in the regression slope.

In planktonic crustacea such as copepods, there is a pronounced antennular aesthetasc sexual dimorphism following the moult from the fifth copepodid male to adult male (Boxshall et al. 1997, Huys and Boxshall 1990, Ohtsuka and Huys 2001). In copepods this moult marks the onset of sexual maturity and a reduction in feeding appendages. It is widely believed that this represents an increased investment for searching strategies related to mate location leading to reproductive success (Ohtsuka and Huys 2001). The acquisition of additional aesthetascs in calanoid copepods also correlates with the move to a pelagic habitat (Boxshall and Huys 1998). Enhanced olfactory sensitivity has been linked with the increased importance of chemical cues within this environment (Ohtsuka and Huys 2001).

Sexual dimorphism in olfactory abilities in invertebrates has been demonstrated to be of particular importance in mediating mate location from a distance and is well reflected in the differential olfactory ability of the sexes in insects (Hallberg et al. 1997, Hansson 1995). Insects show antennal dimorphism most conspicuously in relation to the remote detection of mates by olfactory sex-pheromone signals (Rospars 1988). Antennal dimorphism can result from a larger and more complex shape, an increase in density of chemosensory sensilla or because different sensilla occur in both sexes (Rospars 1988). From the literature published on decapod crustacea there are only two reports of pronounced sexual dimorphism in the antennule aesthetasc number even though a large body of work exists studying these olfactory systems (Gleeson 1982, Hallberg et al. 1997, Mead 2002, Mead 1998, Mead and Koehl 2000, Sandeman and Sandeman 1996, Steullet et al. 2000). The observed sexual dimorphism in antennule aesthetascs of penaeid shrimps indicates a role for olfaction in the reproduction of this species. Although an increase in the number of aesthetasc would not necessarily provide males with increased discrimination for a wider array of signals it would suggest an increase in sensitivity.

Over a wide size range of sexually mature prawns the number of aesthetascs is greater in males than in females. Under the aesthetascs lie a small cluster of olfactory receptor neurons whose dendrites extend up into the aesthetasc to make contact with the external environment. Although each aesthetasc will have a cluster of olfactory receptor neurons underneath, it gives a quantitative method of comparison. The dimorphism observed for the aesthetascs was also documented in this study in the olfactory lobes of the brain. The antennules contain aesthetascs that provide housing for the dendrites of the olfactory receptor neurons which terminate as the olfactory lobes in the brain where chemosensory input is receive and the signal interpreted. While an increase in the number of aesthetascs does not infer that the male prawns have increased range of chemoreception, it does provide evidence that adult males have a greater sensitivity to compounds in the sea water. The dimorphism in the olfactory lobes supports this increased sensitivity with a larger neuropil required to process information being received from a larger number of olfactory receptor neurons.

The antennal, or 'green', glands are situated on each side of the anterior end of the cephalothorax and composed of a coelomosca, labyrinth and urinary bladder, with their ducts opening at the bases of the antennae. The antennal gland has been comprehensively described in the Kuruma prawn, *Penaeus japonicus* (Nakamura and Nishigaki 1991, 1992). An exocrine-like organ has also been described that is adjacent to the nephropore in the crab, *C. maenas* (Fontaine et al. 1989). Masses of rosette glands, lateral and medial to the urether, have also been found in male and female lobsters (*H. americanus*) and have been suggested as possible sources of a pheromone (Bushmann and Atema 1993, 1996). Although no definitive evidence was produced confirming a pheromone function it was shown that the nephropore rosette gland contained proteinaceous compounds and mucopolysaccharides but no phenoloxidase. Crude extracts of these glands only resulted in general non-specific behavior. As described in Objective 2 we were not able to demonstrate the presence of a pheromone candidate from rosette gland extracts.

In collecting information on the components of the olfactory centre we have seen a clear sexual dimorphism in olfaction. Sexual dimorphism has been demonstrated in the number of olfactory receptors and in the olfactory lobes of the brain that are responsible for the processing of olfactory information. The presence of the putative pheromone gland, the rosette glands, has also been demonstrated in penaeid prawns indicating that they do produce pheromones.

ELECTROPHYSIOLOGY

The reason for the lack of action potentials observed, even from known chemo-stimulatory feeding attractants, is unknown. A similar approach was used on

Homarus americanus using feeding attractants as stimulatory chemicals, resulted in recordable electrophysiological changes (Shepheard 1974). Shepheard also had to record from numerous nerve bundles and the responses observed were multiunit recordings in the antennule nerve. One possible explanation is that the low resistance shunt, due to seawater electrolytes, between the two recording electrodes, using the approach we used, did not allow potentials to be observed. Shepheard was able to isolate the antennule nerve in a small volume of oil from that of the stimulant containing seawater by use of a membrane. This difference in electrolyte composition between the solutions undoubtedly assisted in the production of sufficient potentials for recording. Another possible reason is that the antennule nerve in the prawn also has mechanoreceptor, osmoreceptor and baroreceptor inputs, as has been reported for some other crustacea, and their respective activity masked any of that produced by chemoreceptors (Laverack 1964). Another possible reason, as observed by Shepheard (1974), is that antennule preparations were only functional for approximately 10 to 20 minutes at 10°C to 15°C. As recordings on the penaeid antennules took place at the normal water temperature the animals inhabit, i.e. 25°C to 30°C, and took at least 10 minutes to prepare the excised antennules, they may have degraded at a much faster rate and hence the preparations were non-functional by the time recordings were attempted. This study revealed large numbers of axons to be present in the penaeid antennule with axons of diameters of between 0.1 to 0.4 microns and may also explain why it is impossible to record electrical potentials from the receptors of the antennules. Axons of this size will generate minute currents that cannot be seen above the background noise. Whatever the case, it was clear from these attempts that the development of an electrophysiological assays of olfactory receptors using the 'electroantennogram' approach in prawns was not successful. However, single cell patch-clamp electrophysiological recordings has been reported in larger Crustacea with large antennules, as well as by single antennule chemoreceptor nerve cells in short term primary cell culture (Derby 1995, Hatt and Ache 1996). Single unit recordings from the individual receptor cells that are oval and about 45 microns across by 90 microns long, some of which were observed in histology sections of penaeid prawn antennules, might allow single unit recordings. However, these specialised approaches were not available for this study but their application may prove fruitful in future studies. This study was unable to produce any electrophysiology evidence, either for or against pheromones, as recordings of any kind could not be obtained.

WHOLE ANIMAL BIOASSAY – ODOR TRACKING

Penaeid prawns do not demonstrate any stereotyped reproductive behavior that readily lends itself to direct observation. They do not mate guard to any degree although it is possible that if males do locate a female that is about to moult they

may remain in her immediate vicinity. However, even this transient form of mate guarding has not been reported. In stark contrast, crabs and lobster, both display well-defined and distinct reproductive behavior that is readily distinguishable (Bamber and Naylor 1996). The only directly observable reproductive behavior in penaeid prawns is that associated with mating itself and this only occurs over a brief period (Primavera 1979). In closed-thylecum penaeid prawns most phases of copulatory behaviour occur within the water column and is limited to the interval at which the female is still soft following a moult. Mating generally takes place at night immediately following moulting of the females. Wassenberg and Hill (1984) collected data on time of moulting for *Penaeus esculentus* in the laboratory and in the field. Results indicated that they moulted on average 4 hours after sunset (Lab: 4.1 h $\pm$ 2.8 h s.d. Field: 4.0 h $\pm$ 2.9 h s.d.). Males that are attracted to immediate post-moult females are hard shelled (Primavera 1979). When the female is soft the male can insert the spermatophore into the seminal receptacle. Females show distinctive post moult swimming movements (Primavera 1979, Dall et al. 1990). Immediately upon moulting the female swims into the water column and is approached by one to as many as three males, trailing behind the female as she swims (Primavera 1979, Dall et al. 1990). Eventually males will swim in parallel with her and shortly afterwards the female is impregnated with a spermatophore. Mating in penaeids can last a variable length of time, from between 2 and 120 mins (Dall et al. 1990, Primavera 1979). In *P. japonicus* one male followed one female, there was not multiple males chasing female. Male *P. japonicus* only copulated once a night, unlike other crustacea that can copulate multiple times. Hudinaga (1942) reported that male *P. japonicus* pursuit of the female is initiated some time in the late premoult, shortly before the actual shedding of the exoskeleton. In open thelycum species mating has been observed when the male and female are within the intermoult period. As there is little pre-mating behavior in closed-thylecum penaeid prawns a bioassay based on distinct and readily observable behaviour is not possible. The only option for a bioassay directive pheromone purification strategy is the use of an 'olfactometer' assay using odor tracking as the primary indicator on whether a reproductive pheromone is present or not.

To optimise the likelihood of success of a reproductive pheromone bioassay the prawns must be in a reproductive state. Successful mating depends on a complicated cascade of physiological and behavioural events such that various sources of stimulation are involved at several different stages of a behavioural sequence that culminates in copulation. These include sexually mature adults being in a receptive state and in the case of solitary species, or in gregarious species where one sex becomes solitary before mating, one sex seeking out the other. Depending on the species, mate searching may take place some days before an individual is actually capable of copulation or fertilization, which is typically around the time the female moults. The breeding season of penaeid prawns varies but is typically during the

spring and summer months (Dall et al., 1990. Due to closed seasons of the commercial fishery and hence supply from trawlers, this study did not always have prawns that were necessarily of known reproductive status. However, maintenance, or induction, of prawns into a reproductive state was assisted by holding them under spring-like conditions. All trials were conducted in prawns that were held in artificially heated water between 28^{o} to 29^{o}C and under photoperiods simulating spring and summer photoperiods. Nevertheless, remnants of their biological rhythms controlling reproduction may have resulted in some test animals being in a non-reproductive state. Such animals would not respond to reproductive stimuli. As reproductive status is difficult to determine in penaeid prawns, it was not possible to confirm whether any one particular prawn was in a fully reproductive state, however, every effort was made to maximize the likelihood of that being the case.

This study did demonstrate that penaeid prawns can odor track a feeding chemo-attractant to a point source. In the tests in the bioassay arena, the putative pheromone release rate from the female was unknown. A problem with arena aquaria tests is the lack of control for pheromone concentration in the water. A classic chemo-klinokinetic reaction to a chemical stimulus is an increased frequency of turning (searching) in low concentrations and a decreased tendency of turning in high concentrations (Dunham 1978). An equivalent phenomenon has been observed in work on moths (Shorey 1973).

Knowledge of the release rate or concentration of a pheromone is critical to the success of a whole animal odor tracking bioassay (Zimmer-Faust 1989). Crustacea may only show behavioural response to chemical stimuli within a narrow concentration. For example, feeding attractant odors that have characterized chemical identities, or complex chemical mixtures from known sources, can be tested from standards of known concentrations (Carr and Derby 1986b, Zimmer-Faust 1993). Whereas a response to odors is typically a sigmoid-shaped dose/response curve, marine organisms typically only respond to chemicals in the environment over a very narrow range of concentration and often over less than one order of magnitude of concentration from the optimum where responses may fall to background levels (Forward et al. 1987, Browne et al. 1998). The concentration of an odorant or pheromone perceived by an organism is not only influenced by the absolute molar concentration (molecules/L) but also by the periodicity of release rate and the resultant dilution of the compound by hydrodynamics. This determines the concentration experienced at the organism's receptors. In *C. sapidus*, an equivalent behavioral response to prey odorants is obtained from either low volume flow concentrated odor or high volume flow dilute odor (Commins 1995). Similarly, odor released at low rates, either by low volume flow or low concentration, results in a diminished rates of success in searching behaviour (Finelli et al. 2000). Searching success was directly proportional to ambient current speeds with a decline in search

success in currents below 1 cm/s. Without knowledge of the concentration of the putative pheromone in the water column the lack of response from this study may have been due to sub-optimal concentrations at the time of video recording for odor tracking behavior.

In general, the most convincing evidence for reproductive pheromones is in solitary species that mate immediately after the female moults, as is the case in penaeid prawns. It is uncertain at what distance a prawn might be able to detect and successfully search for a point pheromone source. In crabs, the success rate in searching for the source of a food odor attractant was greater when the source was 0.5 m away compared to 1 m (Weissburg and Zimmer-Faust 1993). The release of a reproductive pheromone, like that of a food odor attractant, is most efficiently done from a stationary point source, i.e. the transmitter remains stationary while the receiver searches. As part of their normal nocturnal activities prawns search for food and can cover a large search area. If prawn behaviour in the tank experiments can be extrapolated to the situation in the wild, then general searching behavior is very high in prawns. Even if prawn population density is low, encounter rates with specific odor plumes is probably high as long as there is intense searching activity in general. Such behavioural traits suggest that it would be possible to attract significant numbers of prawns to a point source of a chemo-attractant, such as a reproductive pheromone.

The pheromones involved in far-field attraction in mate searching, involving antennule chemoreceptors may differ from those involved in near-field attraction, involving chemoreceptors on the pereiopod dactyls and mouthparts (Ache 1982). For example, in the amphipod crustacean, *Microdeutopus gryllotalpa*, males search out females secreting a pheromone from a distance but only exhibit additional reproductive behaviour once the male physically makes contact, possibly sensing contact pheromone-like compounds or receiving the same pheromone at a significantly higher concentration, resulting in stereo-specific behaviour and eventual mating (Borowsky 1991). The video analysis of penaeid prawns used in this study when males and females were held within the same bioassay arena offers evidence that a contact pheromone may exist in these prawns. Overall, the complete reproductive repertoire may only be expressed when several sensory inputs are appropriately stimulated. The question on whether a reproductive pheromone would be sufficient to entice an adult male, not only from a distance but also into a trap, remains unanswered at this time.

OBJECTIVE 2
To develop methods suitable for extracting and isolating pheromones from prawns

Introduction

More than 3,500 pheromones have been identified, the majority of which are from insects (Bentley and Watson 2000). In the terrestrial environment these pheromones nearly universally have high vapor pressures, are of low molecular weight, hydrophobic and volatile organic compounds that readily form aerosols and disperse over long distances in air. Due to the requirement for gaseous volatility, there are strong chemical structure constraints on molecular design (Zimmer and Butman 2000). The isolation and identification of signal molecules with the above attributes by gas chromatography and mass spectrometry of terrestrial pheromones are well established. In contrast, an aquatic medium has drastically different physical and chemical properties where aqueous solubility, imparted mainly by electronic charge or hydrophilicity, place vastly different constraints on the type of molecular design. As a result a wide range of chemical types have been implicated as putative chemical signals in the marine environment, including amino acids, biogenic amines, peptides, glycoproteins, purines and steroidal hormones (Zimmer and Butman 2000).

Over 2,000 secondary metabolites acting as chemical defenses have been chemically characterised from marine organisms (Hay 1996, McClintock and Baker 2001). Most of these are small molecular weight non-peptide molecules that have been extracted from mass volumes of biological material by organic solvents, such as methanol or dichloromethane. Typically these are then separated by hydrophobic-interaction or reversed phase HPLC and gas chromatography with their structures identified by mass spectrometry and nuclear magnetic resonance (NMR).

In contrast, purification and characterization of pheromones, and other relevant molecules that are released from the animal into the environment, i.e. exocrines, has met with limited success because many of these chemical molecules are unstable, adsorbed or are bound to other molecules or present in only trace amounts (Zimmer and Butman 2000). Pheromones in the marine environment include those involved in feeding attraction and deterrence (Zimmer-Faust 1989, Leonard et al. 1999), aggregation and school formation (Hamner et al. 1983, Ratchford and Eggleston 1998), habitat selection (Morse 1991, Pawlik 1992), settlement (Tamburri et al. 1996), gamete release (Zeeck et al. 1998), courtship (Breithaupt et al. 1999) and mate searching (Bentley and Watson 2000). Some of these signals involve communicating over short distances, millimetres to centimetres, while others

involve distances over many metres and such differences place specific demands on the molecular nature of the chemicals employed.

Some progress has been made on the identification and characterization of the chemical molecules involved as a mating pheromone in polychaete worms (Zeeck et al. 1994, Hardege et al. 1996) and fishes (Dulka et al. 1987, Sorensen 1992). In the polychaete worms the principal pheromone(s) includes terpenes and other hydrocarbons whereas in fish the female gonadal steroids, and their metabolites, produced by an ovulating female, act as the primary attractants. The majority of pheromones that have been chemically identified and characterized are gamete attracting and mating pheromones. These gamete attractants only operate over very short distances of microns to millimetres and may be more appropriately classified as contact pheromones (Boland 1995). However, evidence for pheromones acting as attractants across significant distances have been described (Dunham 1988, Ingvarsdottir et al. 2002).

Marine organisms can distinguish either novel compounds or unique blends of common substances in complex mixtures carried in the water column (Carr 1988). The most comprehensive studies of chemical communication in the marine environment are on feeding stimulants and attractants released from potential prey (Derby et al. 1996). Of those examined so far, these mixtures are composed primarily of amino acids as well as quaternary ammonium bases, nucleotides, nucleosides and organic acids (Carr and Derby 1986). Whereas feeding stimulants and attractants may operate over relatively long distances, they are not necessarily species specific as reproductive pheromones must be if successful fertilisation is to occur. However, some are species specific and includes the alarm pheromone, anthopleurine, of the sea anemone (Howe and Sheikh 1975).

A range of chemical substances have been tentatively identified as possible pheromones in crustacea, both hydrophilic and lipophilic. All are small molecular weight molecules. The earliest demonstration of a species specific reproductive pheromone in crustacea was in the crab, *Portunus sanguinolentis* (Ryan 1966). The chemical involved was shown to have a molecular weight of 1,000 Da or less (Christofferson 1970). Soon afterwards McLeese (1970) working on the lobster (*H. americanus*) and Kittredge and colleagues (1971), working on several species of crabs, identified a pheromone-like compound. The compound induced typical precopulatory behavior in males. They were able to induce this behavior upon exposing crabs to the lipophilic steroid ecdysteroid moulting hormone, originally termed crustecdysone, at concentrations as low as 10-8 to 10-13 M, but it was not species specific. Many other studies followed on the putative role of the ecdysteroids as reproductive pheromones on other Crustacea but the results were negative (Atema and Gagosian 1973, Cagosian and Atema 1973, Hammoud, Compte and

Ducruet 1975, Dunham 1979, Siefert 1982, Gleeson et al. 1984). It is likely that the ecdysteroids do play a role as a contact pheromone indicating to a male that a female is approaching moult but it is unlikely that it is acting as a species specific pheromone attractant. A copulation pheromone has been described in the crab, *Telmessus cheiragonus*, but its chemical nature has not been fully characterised other than it is a water-borne pheromone with a molecular weight of less than 1 kDa (Kamio et al. 2002). Overall, however, the technical problems associated with chemical characterization are significant and the complete chemical characterization of several putative crustacean pheromones remains unknown. Isolation and characterization is further hampered given that a robust behavioural assay that will reliably indicate the presence of a pheromone in the water column is often lacking.

APPROACHES TO PURIFICATION, ISOLATION AND CHARACTERIZATION

The key prerequisite for reproductive pheromones (also known as sex pheromones) are that they must be species specific. The unique molecular structure needs to be transmitted over significant distances and have a half-life sufficient to allow the molecule to be detected by the opposite sex while at the same time eventually degrading so that it no longer operates as a pheromone. Unique chemical structures are readily produced from polymeric systems with a limited number of repeating units. For example, while there are only 20 primary amino acids in living organisms these are over 3.2 million unique polymeric peptides that can be created from peptides of only 5 amino acids in length. A high percentage of peptides in estuarine waters are <10 amino acids in length (Coffin 1989). In addition, in the marine environment, peptides are water-soluble, due to the charged nature of the terminal primary amine and carboxylic acid groups at near neutral pH (seawater is pH 8.5), and are not volatile. As peptide production is common to all prokaryotic and eukaryotic life any organism could create unique molecules. Taken together, peptides are particularly good chemical candidates that could potentially form a vast array of species specific pheromones in the marine environment (Rittschof 1990). On the other hand, peptides are also readily degraded over time by extracellular proteases, for example from bacteria, and hence have a limited half-life in the marine environment (Hoppe 1991). Bacteria also have a very high surface area to volume ratio and a high-affinity transport system with an efficient osmotrophic uptake of small molecules (Karp-Boss et al. 1996). In addition, peptides containing fewer than five amino acids can be absorbed directly by microbes without prior extracellular hydrolysis (Payne and Smith 1994). Hence, although peptides may be attractive molecular candidates as marine pheromones, there are significant disadvantages. However, small molecular peptides containing basic carboxyl terminal residues are taken up significantly slower (up to 3.5 times) than either free amino acids or non-basic residue peptides (Decho et al. 1998). Hence, small

molecular weight peptides of 3-10 peptides which contain basic amino acid residues remain attractive candidates as highly soluble molecules acting as pheromones (Browne et al. 1998). However, not all reproductive pheromones characterized have been based on the polymeric structure of peptides however. In goldfish, steroids and their metabolites act as reproductive pheromones (Sorensen 1992, Stacey et al. 1994). The chemoreceptors of these fish are highly sensitive and specific, with minute changes in molecular structure of the steroid resulting in hundred-fold increases in nanomolar threshold concentrations (Sorensen and Caprio 1998). A reproductive pheromone from the Hair crab (*Erimacrus isenbeckii*), which induces precopulatory guarding behaviour, has been purified and characterized as a novel ceramide (Asai et al. 2000). However, it is questionable whether ceramides are released from the female or are purely contact pheromones. Although a contact pheromone may have species specificity it does not necessarily have the molecular properties to be transmitted through the water column.

SOURCES OF REPRODUCTIVE PHEROMONES

Pheromones for purification, isolation and characterization can be obtained in several ways. Perhaps the most appropriate, in terms of biology, is to collect the pheromone sample once it has been fully processed and released into the environment by the organism. Although the pheromone is fully active in this form it can only be collected when it is known to be present. Ideally this requires a bioassay test when the receiving organism displays the appropriate behavioural response but this is usually when the pheromone is most dilute. From a chemistry standpoint, the pheromone has to be concentrated by many orders of magnitude from its dilute state by various purification methods for chemical analysis. The co-concentration of other molecules in the seawater sample can overwhelm the purification and isolation process of the pheromone and poses a multitude of challenges.

A second approach is to excise the tissue or organ from the animal that is the source of the pheromone. However, the source of the pheromone is not necessarily known and may be based mainly on speculation. In addition, the tissue or organ may not store the pheromone before release and hence not be an enriched source of it. Even if the pheromone is stored in high concentrations it may not be in its biologically active form as many chemical communication molecules, such as hormones (endocrines) and pheromones (exocrines) are chemically modified to its biologically active form at the time of release (Laufer and Downer 1988). If the putative pheromone is isolated in this form there is no way to confirm its presence using a bioassay as it will not be reactive. Nevertheless, the purification and isolation of a pheromone from its source remains a possibility.

A third approach is to collect the pheromone at the moment of release from the organism. This approach requires accurate knowledge of the production site and release site of the pheromone and is most readily done if it is discharged from a point source. It may also require constraining the animal but the stress of doing so may interfere with the animals' normal pattern of behavior. As a compromise the animal may be contained in a small volume of seawater and the release rate of the putative pheromone monitored. If an accelerated release rate of the pheromone is detected the 'enriched' water sample may be collected for purification and isolation.

SEAWATER

The initial purification of a putative pheromone from seawater requires its concentration. The most appropriate concentration method to use is solid phase extraction. This is an extraction technique based on the selective partitioning of one or more components between two phases; one phase is a solid sorbent and the second is typically a liquid, but is may also be a gas or a supercritical fluid. The components of interest may either preferentially absorb to the solid or they may remain in the liquid. Once equilibrium has been reached the two phases are physically separated by decanting, filtration, centrifugation or a similar processes. A particular adsorbed molecule can be desorbed by eluting with an appropriate solvent. To isolate specific components a series of solvents or solvent mixtures of successively increasing elution strength is used to selectively elute the retained molecules (Pempkowiak 1983, Vastano et al. 1987). This approach is widely used as a first step in the isolation of active pheromones (Blight 1990, Forward et al.1987).

The three classes of solid phase extraction sorbents include a class primarily used for adsorption by hydrogen bonding or dipole-dipole interaction to absorb analytes from non-polar solvents. Aminopropyl (NH2) and cyanopropyl (CN) modified silica are appropriate as they are more compatible with an aqueous medium. These adsorbents are widely used for extraction of phenols, petroleum fractions, drugs and drug metabolites. The second class is modified to be hydrophobic, and are classed as reverse phase sorbents, which themselves are available in four subclasses, C18, C8, C4 and C2 formats, ranging from strongly hydrophobic to low hydrophobicity. These sorbents are widely used for the extraction of general metabolites in biological fluids, drugs, peptides and proteins, trace organics and organic acids. The third class is modified to be hydrophilic and may be either a strong anion-exchanger or weak cation-exchanger. Both have a large pore size for the extraction of either anionic or cationic analytes and are excellent for the extraction of peptides and proteins, and other applications including pesticides, herbicides and steroids. These three classed of adsorbents are capable of extracting known classes of environmental molecules. Unless the putative pheromone is of a highly volatile nature, solid phase sorbents are

capable of extracting a putative pheromone of unknown chemical characterization from seawater and retaining it for later elution and analysis (Kieber and Mopper 1990).

Once a solid phase extraction matrix has been identified which binds the pheromone and allows successful elution and isolation the pheromone candidate requires isolation. The main approach for the isolation of the pheromone chemical nature is high performance liquid chromatography (HPLC) (McCaffery and Wilson 1990, Burlingame and Carr 1996, Larsen and McEwen 1998). The same solid phase extraction matrix can be utilised in a HPLC format that allows a much higher resolution separation technology to specifically isolate the candidate pheromone from other contaminants. Purification progress can be followed with several detector formats to identify the most appropriate one (Johnson and Stevenson 1978, Snyder et al.1988). In the first instance ultraviolet-visible (UV-Vis) spectrometry detector is most commonly utilised (McCaffery and Wilson 1990). Specific fractions from these chromatographic isolations can be tested again in the experimental observation tank to confirm the presence of pheromone and retention of biological activity.

The final stage includes characterisation of the candidate pheromone by mass spectrometry. Since the chemical nature of the pheromone is initially unknown the candidate pheromone can be examined by mass spectrometry, such as Fourier transform ion cyclotron resonance mass spectrometry (FTMS) (Larsen and McEwen 1998). Analysis by the FTMS allows accurate mass determination of the substance. The use of an additional capability of the FTMS by mass spectrometry - mass spectrometry (MS/MS) allows some characterisation of the chemical substance. For complete chemical characterisation of the substance, however, nuclear magnetic resonance (NMR) studies are required to complete the chemical and structural characterisation. The use of NMR requires a minimum of microgram quantities of purified substances to complete the analysis. The HPLC-mass spectrometry-NMR approach has recently been used successfully to purify, isolate and characterize novel ceramides from crabs that have been shown to act as reproductive pheromones (Asai et al. 2000, Asai et al. 2001).

URINE

Reproductive pheromones are believed to be released in the urine from the antennal gland in the shore crab (*Carcinus maenas)*, the crab (*Portunus sanguinolentus)*, the blue crab (*Callinectes sapidus)*, the lobster (*Homarus americanus)* and the crayfish (*Astacus leptodactylus*) (Eales 1974, McLeese et al. 1977, Christofferson 1978, Gleeson 1980, Atema and Cowan 1986, Breithaupt and Atema 1993, Breithaupt et al. 1999, Breithaupt and Eger 2002). Although the antennal gland may have a urinary bladder its capacity is limited. In the Kuruma prawn the urinary bladder is not

tubular but vesicular and appears not to have any muscular system that can regulate urine flow (Kakamura and Nishigaki 1991). In contrast, in crabs and lobsters, urine is believed to be injected under pressure from the urinary bladder through the nephropore into the bilateral gill current which results in transport of the fluid away from the animal and hence can transmit chemical signals significant distances into the environment (Bushmann and Atema 1996). Methods have been developed for crabs and lobsters for the direct collection of urine from the bladder. Urine can be obtained from crabs that are constrained, and, after drying the third maxillipeds and blocking the gill cavity openings, a fine pin can be used to lift the operculum cover of the nephropore and urine flow expressed (Bamber and Naylor 1997, Kamio et al. 2000). Urine released can be collected from freely moving crabs and lobsters by catheterising them (Holliday 1977, Lindstrom 1991, Breithaupt, Lindstrom and Atema 1999). Besides allowing the animal to interact normally with its environment, the catheterising method allows for the measurement of urine output. In this way it has been demonstrated that lobsters readily change urine flow, both in volume and frequency, during social interactions (Atema and Cowan 1986, Cheng and Chang 1991, Snyder and Chang 1991, Snyder et al. 1993). In a further modification of the technique Breithaupt and Eger (2002) used the dye Fluorescein by which to visualise the release of urine by lobsters. This approach could be used in prawns in order to collect seawater from a aquaria immediately after urination bouts. In this way it might be possible to obtain a seawater sample that is enriched with putative pheromone. Although it is believed that pheromones are released in the urine during these interaction the chemical nature of the molecules is unknown.

Methods

IN-LINE CHROMATOGRAPHY OF 'FEMALE' SEAWATER

The specially designed aquarium tank for the 'olfactometer' bioassays (see Objective 1) was used for attempts to purified putative female pheromones. A female that was predicted to moult was placed in a holding tank with flow through water that fed into either channel 2 or 3. A non-moulting female was placed into the remaining holding tanks flowing into either channel 2 or 3. Male behavior in the tank was monitored as described in Objective 1. A proportion of the water flowing through the tank containing the female predicted to moult was passed through a series of solid phase sorbents (Fig. 2.1). The chromatography columns were plumbed so that the female seawater first passed through a 3 and 1.3 micron pre-filter to remove particulates. The seawater then passed through three XK 50/20 (Pharmacia), 50 mm internal diameter chromatography columns plumbed in series packed with different solid phase matrixes (Coll et al.1982, Burke 1984, McDonald and Bouvier 1995). Seawater first passed through a Macro-Prep ceramic hydroxyapatite column (Bio-Rad, Cat. no. 157-0080) to extract peptides and proteins from the seawater, then through a AG50W X-2 strong cation exchanger to remove salts and finally through a Bio-Bead

SM-2 column to extract non-polar substances. All solid phase chromatography columns were prepared according to manufacturers instructions except for Bio-Beads SM-2 as described below.

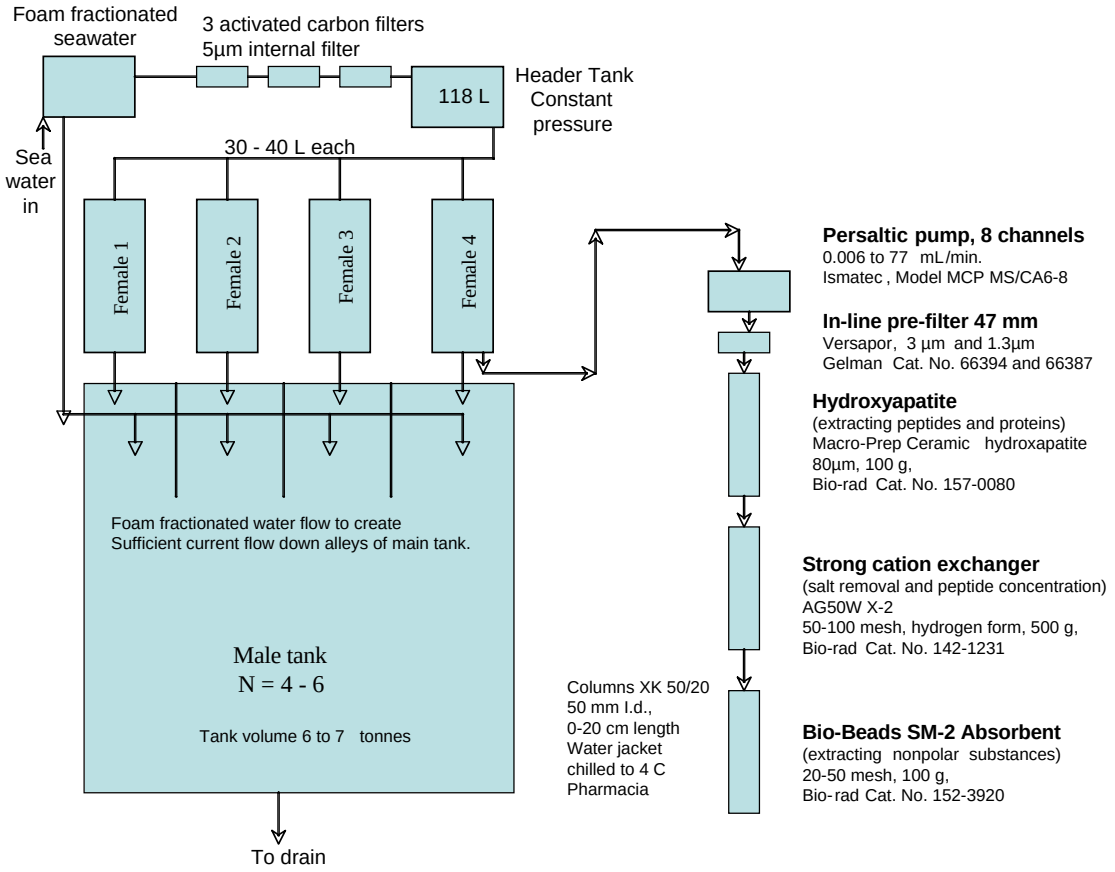


Figure 2.1. Schematic diagram of the flow-through aquarium system set-up for simultaneous extracting a proportion of the flow through water through a series of chromatography columns for chemical purification and video monitoring of odor tracking response in males to the presence of female pheromone release.

Elution of the Macro-Prep hydroxapatite column was performed by eluting with a linear gradient from 100% seawater to distilled water followed by 10 mM sodium phosphate, pH 6.8 and a linear gradient to 250 mM sodium phosphate, pH 6.8 at a flow rate of 6.5 mL /min. No elution of the AG50W X-2 strong cation exchanger was made as its role was to deionized the seawater in preparation for passage through the Bio-Beads SM-2 column.

Bio-Beads SM-2 (Bio-Rad Laboratories) are non-polar polystyrene adsorbents for the adsorption of highly dilute nonpolar (water soluble) substances from aqueous solutions. Bio-Beads SM-2 (15 grams) were added to a 50 mL capped plastic centrifuge tube followed by the addition of 30 mL high HPLC grade methanol. The

tube was capped and the contents thoroughly mixed. The tube was allowed to stand at room temperature for a few minutes to allow settlement of the beads. The methanol fraction was decanted and discarded. The washing (mixing)/decanting process was repeated three more times. On the last wash the Bio-Beads with 30 mL of methanol was uncapped and placed in a vacuum desiccator. The Bio-Beads/Methanol suspension was degassed for 5 minutes using a mechanical diaphragm vacuum pump, and afterwards recapped ready for use.

A chromatography column of Bio-Beads was made by fitting a 200 micron polyethylene frit (Alltech, Cat. no. 211775) into the bottom of a 75 mL reservoir column (Alltech, Cat. no. 210575) with HPLC grade methanol and allowed to drain to waste. A plastic tube was attached to the column outlet and the tubing closed by pinching with a clamp. Previously washed and prepared Bio-Beads were degassed immediately before use and thoroughly mixed in their storage vial and the entire slurry suspension was quickly poured into the column. The bottom clamp was opened and the methanol allowed to drain away but only to the surface of the bed of Bio-Beads and the outlet re-clamped. Another polyethylene frit was fitted to the top of the Bio-Beads to seal the bead bed. The tube headspace was filled with Milli-Q water and an inlet head fitted to the top of the chromatography column and the inlet tubing connected to a peristaltic pump. The column was washed with 100 mL of Milli-Q water through the column at a flow rate 10 mL/minute and the effluent monitored by spectrometer. Five volumes of HPLC grade 100% methanol were pumped through the column to elute adsorbed compounds. After each extraction of seawater, the Bio-Bead columns were rinsed with 250 mL of fresh Milli-Q water at 10 mL/min. The final drops of water from the Bio-Beads were removed from the top of the porous frit positioned at the surface of the resin bed. The Bio-Beads were transferred to a 50 mL tube and 30 mL of HPLC grade 100% methanol added, the tube was capped and vigorously shaken. The Bio-Beads were allowed to settle and the methanol decanted. The tube was filled with HPLC grade 100% methanol, capped and stored at room temperature for subsequent extractions.

Water samples were collected from tanks in which moulting and non-moulting female *Penaeus semisulcatus* had been held. The water samples were stored for extraction using different protocols. The aim was to investigate time of collection and extraction on the isolation of possible pheromones from seawater. Female *P. semisulcatus* were collected late in the afternoon from the main holding tank and transferred to a static 60 L tank containing 20 L of seawater with aeration. Three water types were collected by the in-line chromatography system; seawater only, as a control, seawater from a female that had moulted the previous night and seawater from a female that had not moulted.

BATCH CHROMATOGRAPHY OF 'FEMALE' SEAWATER

The short-term holding aquarium system was designed specifically for the collection of pheromones released by prawns of a known moult stage (Fig. 2.2). After the tagged female prawns in the main holding tank were checked for moult status, pre-moult females were selected and transferred to a 40 L tank containing a static seawater volume of 20 L, with aeration, on the anticipated night of moult. Adult *P. monodon* moult approximately every 16.4 days and estimates for the date of moult of *P. semisulcatus* were based on this (Hall et al. 2003). The seawater used to fill these tanks was processed as described as for TAF water in general and further treated by passing the seawater through three activated carbon filters and a 1 µm nominal filter. The prawns were held in these tanks overnight, with aeration but without food, for up to 12 hours. The following morning a 2 litre sample of water (1/10 total) was collected into clean, 2 L high-density polyethylene bottles (Nalgene, 2007-0064) and 200 mg of sodium azide added as a preservative with stirring to each 2L bottle. The bottles were capped, stored at 40°C and treated up to methanol phase (see below) within 24 hours.

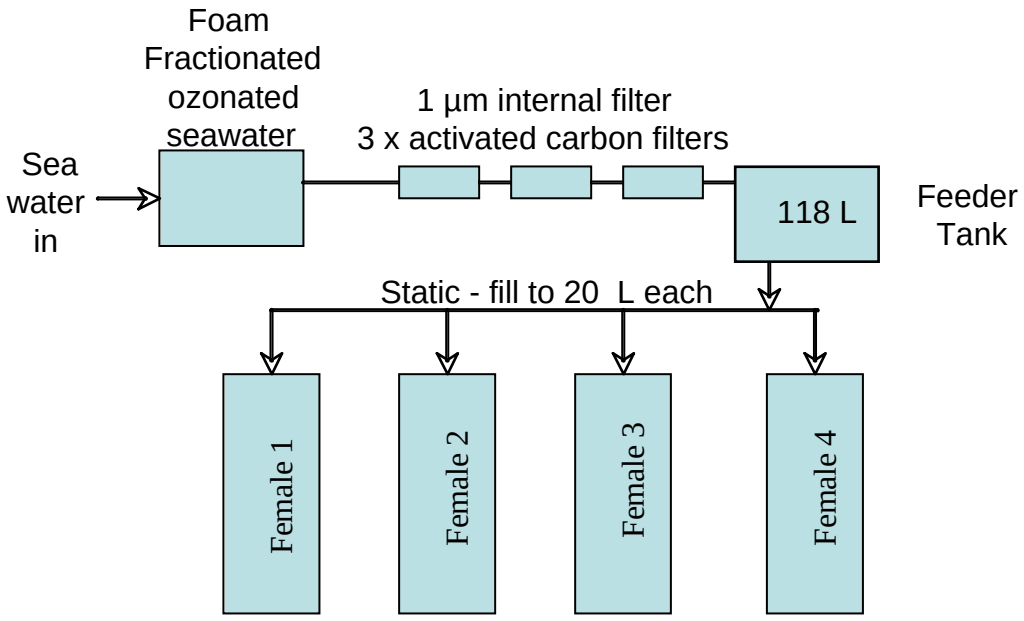


Figure 2.2. Schematic diagram of the static aquarium system set-up specifically for the collection of putative pheromones from 1/10 the total volume of batch static seawater samples for tanks holding females from specific physiological states.

A reverse phase chromatography column was constructed that could purify compounds over a wide range of polarities. Individual 10 gram C-18 Sep-Paks (Alltech, cat. no. 235410) were connected to a solid phase extraction manifold. Each cartridge was washed with 5 individual 30 mL portions of HPLC grade 100% methanol with care taken

not to allow the resin bed to dry out. The cartridge was then washed with 30 mL of Milli-Q water and removed from the solid phase extraction manifold. The cartridge headspace was filled with Milli-Q water and connected to a peristaltic pump.

The low molecular weight molecules in the 2 L seawater samples were adsorbed onto the solid phase matrix by connecting the outlet tube of the peristaltic pump to a 47mm O.D. inline 1.3-micron prefilter (Gelman, Cat. no. 66387). The prefilter outlet was connected to the top of either the Sep-Pak extraction cartridge and the sample pumped through the cartridge with the peristaltic pump set at 10mL/min, allowing approximately 4 hours for the entire 2 litres of sample to pass through the extraction cartridge. When the entire sample had passed through the extraction column the pump was stopped and the end-cap removed. The remaining water sample from the extraction tube headspace was allowed to drain and then filled with Milli-Q water. A reservoir of Milli-Q water was placed on the pump inlet tube and purged. The pump outlet tubing was reconnected to the extraction cartridge and 100 mL of Milli-Q water pumped through the extraction cartridge. The pump was then disconnected from the cartridge and the remaining Milli-Q water from the cartridge headspace was allowed to drain away.

Individual solid phase extraction cartridges were eluted by connecting them to a vacuum apparatus. A clean 50 mL tube was placed under the individual extraction cartridge outlets and the remaining water (<1 ml) from the extraction cartridges aspirated into a waste vial. A sample of 50 mL of 80% acetonitrile was slowly aspirated through each cartridge and the sample collected and marked as Fraction 1. Another sample of 50 mL of 100% acetonitrile was slowly aspirated through the cartridge and the sample collected and marked as Fraction 2. The two fractions were decanted into clean scintillation vials and dried overnight on a Savant rotary evaporator. The vial was capped and stored dry at -20^{0}C until analysis.

In preparation for HPLC analysis, individually dried samples of fractions 1 and 2 were dissolved in 750 microliters of 100 % acetonitrile, capped and carefully sonicated with additional shaking for 15 seconds. Each capped sample was incubated at room temperature for 10 minutes. An additional 750 microliters of Milli-Q water was added to Fraction 1 and to Fraction 2 an additional 750 microliters of 50% acetonitrile/water. The samples were briefly vortexed and incubated at room temperature for 15-20 minutes. A 1 mL sub-sample of each fraction was added to a 1.5 mL plastic vial and centrifuge at 10,000G for 10 minutes. The supernatant was removed and added to a new 1.5 mL plastic vial and stored below -20^{0}C until analysis.

Gradient reverse phase HPLC was performed using an Alltima C-18, 4.6mm X 15cm analytical column with Solvent A as 5% acetonitrile in water containing 0.05% trifluoroacetic acid and solvent B as 100% acetonitrile containing 0.05%

trifluoroacetic acid. Flow rate for the pumped mixed solvents was 1 ml/minute. Separations were performed at room temperature.

Two gradient programs were used. The original gradient program (Gradient A) used was:

TIME (minutes)	FLOW (ml/minute)	%A	%B
0	1	100	0
1	1	100	0
30	1	0	100
34	1	0	100
35	0.5	50	50
5 minutes Equilibration	1	100	0

To obtain better resolution the gradient program (Gradient B) was later changed to Solvent A as 5% acetonitrile in water containing 0.05% trifluoroacetic acid and solvent B as 75% acetonitrile containing 0.05% trifluoroacetic acid. Flow rate for the pumped mixed solvents was 1 mL/minute.

TIME (minutes)	FLOW (ml/minute)	%A	%B
0	1	100	0
1	1	100	0
90	1	0	100
94	1	0	100
100	0.5	50	50
5 minutes Equilibration	1	100	0

The HPLC system comprised an ICI LC 1150 gradient pump and a Linear 206 UV spectrometer. Injection volumes of 20 microliters of dissolved/centrifuged sample were injected onto the analytical column. Detection was performed simultaneously at 220 nm (for basic peptides) or 230nm, as well as 254nm and 280nm.

Moulting occurs sometime during the night. Since the exact release time of a putative pheromone is unknown samples were collected at varying times as a negative result might only indicate that the pheromone had degraded by the time of sample collection the following morning and hence no signal would be found. Three samples of seawater from the static tank, or batch chromatography samples, were collected from a tank in which a female had moulted. The first sample was collected immediately after moulting occurred, the second was from a sodium azide preserved sample that had been stored at 4^{o}C until the following morning for analysis, and the third was sampled from the holding tank the following morning.

PHEROMONE GLAND - ROSETTE GLANDS

Prawns were chilled in ice cold seawater and the area of the rosette gland, based on histological evidence was dissected out. The gland was homogenised in 1 mL 2% formic acid in Milli-Q water. Other tissues, including ovarian tissue, epithelial tissue, lymphoid organ and haemolymph were homogenized in 1 mL seawater as controls. The extracts were briefly centrifuged to remove particulates and the supernatant added and centrifuged in prewashed Ultrafree MC membrane filters (Millipore Cat. no. UFC3LGCNB) with a 5,000 molecular weight (MW) cut-off for 60 min at 5,000 g. A 1 mL sample of extract was injected onto a gel filtration size exclusion column (Superdex Peptide HR 10/30 column, Amersham Biosciences) and eluted with 2% formic acid in Milli-Q water at 1 mL/min using a ICI LC1150 HPLC pump which was fitted with a back pressure controlled after the pump but before the column to prevent backpressures of over 1.5 Mpa, which would otherwise compact the porous agarose bead bed of the column. The eluent was passed through a UV spectrometer detector and monitored at 215 nm. The Superdex Peptide column has an optimal separation range of molecules between 100 and 7,000 MW. The use of a gel filtration size exclusion column avoids the uncertainty of whether a particular molecule, whether hydrophilic or hydrophobic, will adsorb onto the solid phase matrix and elute. All substances injected onto the Superdex column will elute off the column regardless of polarity based only on its molecular weight.

URINE

Lobsters release pheromones in their urine and evidence supports the likelihood that prawns would do likewise. Although it is desirable to collect putative pheromone samples from unrestrained unstressed prawns there were significant problems with the recovery of the pheromone after massive dilution in seawater as well as difficulties in purifying it on solid phase matrixes that can only adsorb molecules within limited ionic strength margins. Collection of the actual gland that produces the pheromone, believed to be the rosette glands, is problematic in that the extract containing putative pheromone might not be fully processed and biologically active until it is actually released from the gland itself. Efforts were therefore devoted to the examination of whether it would be possible to detect urination in unrestrained females that would allow the collection of a seawater sample for chemical analysis immediately after the release of the putative pheromone.

Green tiger prawns, *P.semisulcatus*, were maintained as previously described in the whole animal bioassay methods (Objective 1). Animals were moult staged by looking at their uropods in methods described previously (Longmuir 1983; Smith and Dall 1985; Robertson, Bray et al. 1987) where setal development in the uropods or pleopods is used to assess the stage in the moult cycle. A solution of 0.2% sodium Fluorescein in 0.9% sodium chloride (Molecular Probes, Cat. no. F-1300) was injected

into the pericardial sinus of the prawn. The dye was injected using a 1 ml syringe with a 25 gauge needle at a dosage of 2-4 µg/g body weight. Individual prawns were held in a 10 litre tank fed fresh seawater at around 3 L/min from the same system described in the whole animal bioassay trial prior to foam fractionation and carbon filtration. Fluorescein is water-soluble and is highly visible under UV light at extremely low concentrations. The dye is released by usual urination pathways in the prawn. Prawn urination can be monitored by time-lapse photography illuminated under far blue and near UV light in order to visualise the fluorescein dye. Estimates of frequency of urination, and, when putative pheromones would be released into the water column could therefore be visualized in fluorescein injected prawns. However, the time-lapse photography method depended on restraining the female which could adversely affect behavior.

Another approach to determine the timing of urination is to monitor a sub-sample of the aquarium water in which a prawn is held. A method to observe urination rates in unrestrained prawns was developed using a fluorescence detector. Fluorescein is highly fluorescent upon UV excitation with concentrations as low as 5 x 10-11 M being detectable. Female prawns were injected with a single bolus of fluorescein into the pericardial sinus at a concentration of 4 micrograms per gram body weight and released into a 50 L aquarium. Water was sampled from the tank through 0.76 mm internal diameter tubing via a 5µm membrane filter via a peristaltic pump running approximately 1 ml/min straight into a fluorescent detector (GBC Model 1255) set at excitation at 488 nm and emission at 530 nm. Data was collected using an AD instrument Powerlab recorder and Chart (AD Instrument) software. Peaks in fluorescence in the tank were recorded as urination events. Time between urination events was used to compare different prawns in different states of moult. If the female demonstrates a higher frequency of urination at specific times, for example in the moult cycle, it would allow the optimisation of a sampling schedule by which to increase the probability that an 'enriched' pheromone seawater sample could be collected.

A further refinement in collection putative pheromone in as concentrated form as possible involved collecting urine directly either from the urinary bladder by catheter or expression of urine. The catherization and collection of urine has been previously described for crabs and lobsters (Holliday 1977). In these species it is possible to gain direct access to the nephropore, which is the orifice from which urine is discharged. Methods have been developed in lobsters to encircle the nephropore and seal it with an adhesive to allow urine direct access into a catheter (Lindstrom 1991). However, in prawns the maxillipeds overlay the nephropore thereby preventing direct access to it. However, access to the nephropore is possible by restraining a prawn on its back and gently teasing back the maxillipeds. The maxillipeds were dried by blotting the surface with tissue paper and by gently

squeezing the based of the antennules it was possible to express a fluid that appeared to originate from the nephropore. To confirm the orifice at the base of the antennules was indeed the nephropore, serial sections for histological analysis were prepared. As an alternative collection method, a fine capillary catheter tubing was used to gently probe the nephropore orifice until there was expression of urine, which was then collected by suction through the capillary attached to a micro-syringe.

Two methods were used to gain independent confirmation of the nature of the solution collected by catheter. In the first, a single bolus of fluorescein, as described above, was injected and measurements made of fluorescein concentration in various fluids. As fluorescein is preferentially secreted from the body in urine a 'true' urine sample should be concentrated with the dye. The other method was to measure ammonia and nitrate concentrations using a commercial colorimetric kit. As the antennal gland is the organ of nitrogenous waste production, fluids containing high concentrations of ammonia and nitrate would identify the fluid as urine. Urine samples from *Penaeus monodon* (15 samples: 11 females and 4 males), *Penaeus semisulcatus* (56 samples: 25 females and 31 males) and *Penaeus esculentus* (29 samples: 15 females and 14 males) were collected.

PHEROMONE PURIFICATION AND MASS SPECTROMETRY FROM URINE

It is believed that the expression of a female specific pheromone occurs immediately before moult (ecdysis). By moult tagging individual prawns urine samples were collected around the expected time of moult. Samples of urine were collected using a catheter with slight pressure applied upon the opening of the uropore (nephropore). As it was being release urine was vacuumed from the pore stored at –20ºC until processing.

In order to obtain more information on the presence or otherwise of a pheromone-like substance in the urine of penaeid prawns in general, two species were examined, *P. monodon* and *P. semisulcatus*. In the first instance urine samples were processed through a 5,000 MW cut-off membrane and chromatographed on a Superdex Peptide column as described above for gland extracts. This chromatographic method was used as it avoided all the uncertainties of the molecular nature of the putative pheromone and whether it would not only adsorb to the solid phase matrix but whether it would also elute from it. The 5,000 MW cut-off membrane eliminates high molecular weight compounds that can interfere with mass spectrometry preparation. There are no known marine pheromones with molecular weights of over 5,000. The size exclusion chromatography was used to only examine for compositional differences in urine from moulted and non-moulted females in

relation to a molecules of specific mass. This initial chromatographic method allowed the further downstream processing of samples by mass spectrometry.

The efficiency of ionisation in the mass spectrometer (MS) is dependant on total ionisable chemical concentration (Janiszewski et al. 2001). As urine is isotonic with respect to seawater, the ionisable molecules from the salts alone will be in the parts per thousand concentration as opposed to parts per million or billion of pheromone with subsequent lost of signal from the MS detector. However, as the urine is diluted 1/10 and is collected in small volumes it is possible to use RP-HPLC without overwhelming the binding capacity of the column as only a few micrograms of salt are bound, leaving sufficient binding capacity for the pheromones. For mass spectrometer analysis all urine samples were diluted 1:10 in 100% MilliQ water prior to injection. A standard was added to the sample glycine-glycine-histidine (GGH) (monoisotopic mass 270.1) at a concentration of 0.1 mg/mL so that we could normalise the peak intensity of each mass across all runs. Samples were separated by liquid chromatography using a Jupiter 5µ 300Å C18 column (Phenomenex) at 25 C with a gradient from 100% H_2O with 0.1% formic acid to 80% Acetonitrile with 0.1% formic acid (Table 2.1) over 50 minutes at a flow rate of 0.3 mL/min (Agilent 1100 series). This was coupled to a Bruker Esquire 3000plus Quadruple Ion Trap Mass Spectrometer (LCMS). Operational parameters for the Bruker Esquire mass spectrometer was set with a scan range from 220 – 1000 m/z and are summarised (Table 2.2). Base Peak Ion Chromatograms (BPC) were screened for all compounds over 50 minutes, information was recorded as the dominant mass/charge signal (m/z), retention time and extracted peak ion intensity. Peaks were recorded as m/z as some compounds may be present in more than one charge state (+1, +2 or +3). However, for practical purposes m/z is equivalent to the molecular mass of the unidentified molecule. An example of total ion chromatogram with mass spectrum is shown in Figure 2.3. Total ion chromatograms were generated and analyzed (Bruker Data Analysis V 3.1 software) by selecting peaks from the LC trace and analyzing between 220 and 1,000 m/z. Base peak chromatograms were generated for individual m/z and peak ion intensity and retention time recorded. Signals from injections of 0.1% formic aicd in 100% Milli-Q (blanks) were subtracted from unknown urine signals and urine-specific m/z ratios, retention time and peak ion intensity recorded.

Table 2.1. Solvent gradient used on LCMS with Jupiter C18 column.

Time (mins)	100% H_2O 0.1% formic acid	80% Acetonitrile 0.1% formic acid
2.00	100	0
42.00	20	80
50.00	20	80
52.00	100	0

Table 2.2. Operational and acquisition settings for Bruker Esquire 3000plus Quadruple Ion Trap Mass Spectrometer (LCMS).

Mode	
Mass Range Mode	Std/Normal
Ion Polarity	Positive
Ion Source Type	ESI
Alternating Ion Polarity	off
Current Alternating Ion Pol	Positive
Divert Valve	to Source

Tuns SPS	
Target Mass	450 m/z
Compound Stability	100 %
Trap Drive Level	100 %
Optimize	Normal
Smart Parameter Setting	active

Tune Source	
Trap Drive	34.6
Octopole RF Amplitude	150.0 Vpp
Lens 2	-60.0 Volt
Capillary Exit	124.8 Volt
Dry Temp (Set)	330 °C
Nebulizer (Set)	40.00 psi
Dry Gas (Set)	8.00 l/min
HV Capillary	4000 V
HV End Plate Offset	-500 V

Trap	
Rolling	on
Rolling, Averages	2 cts
Scan Begin	220 m/z
Scan End	1000 m/z
Averages	8 Spectra
Max. Accu Time	200000 µs
ICC Target	35000
Charge Control	on

MS/MS Automatic	
Auto MS/MS	off

Fragmentation Options	
CutOff Selection	Default
SmartFrag	On
SmartFrag Start Ampl	30 %
SmartFrag End Ampl	200 %
Fragmentation Width	10.00 m/z
Fragmentation Time	40000 µs
Fragmentation Delay	0 µs

Results

IN-LINE CHROMATOGRAPHY OF 'FEMALE' SEAWATER

Elution and monitoring of fractions from all of the chromatography columns did not reveal any peaks in absorbance at 220, 230, 254 or 280 nm above baseline. However, none of the females that moulted in their respective holding tanks, as described in Objective 1, induced any odor tracking behavior in the males. Nevertheless, it was expected that some pheromone-like substances would have been released by the female and yet no signal was observed. There are several possible reasons for this with the foremost being that the ionic strength of seawater is not compatible with many of the required chemical environment conditions necessary for adsorption of molecules onto the solid phase matrix. For example, although the Macro-Prep ceramic hydroxapatite requires neutral pH for adsorption of proteins (seawater is pH is 8) it also ideally requires low ionic strength solutions, and these conditions aren't met in full strength seawater. As the prawns are marine organisms it is not possible to hold them in water that is sufficiently low in ionic strength to allow adsorption of molecules to the solid matrix while as the same time not stressing or killing them. As all results revealed no putative pheromone signal using this method alternative approaches were adopted.

BATCH CHROMATOGRAPHY OF 'FEMALE' SEAWATER

Analysis of the two fractions from 80% and 100% acetonitrile extraction revealed that all the proteinaceous material eluted off in the first fraction and therefore only this fraction was analysed further. Samples from moulted and non-moulted female seawater samples revealed some peaks that appeared to be unique to moulting females (Fig. 2.3). However, the peaks varied between females and there were no peaks that had similar retention times common to moulted females.

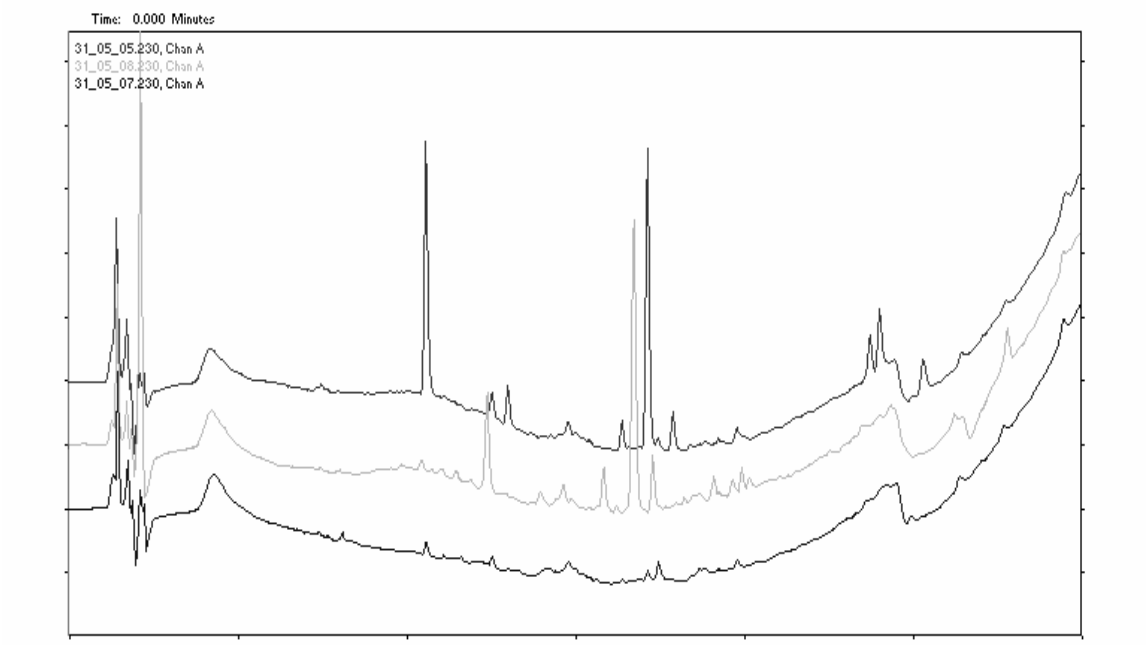


Figure 2.3. Reverse phase high performance liquid chromatography (RP-HPLC) plot of 230 nm absorbing compounds from putative pheromone containing seawater from moulted and non-moulted female *Penaeus semisulcatus* compared to seawater only.

The original gradient program (Gradient A) was slightly modified to program Gradient B to give a more extended gradient over time to give greater separation between the peaks. However, although unique peaks from female seawater were observed they were not consistent between females.

As any putative pheromone must eventually decay, seawater samples of aquarium tanks in which females had moulted were analysed of different times post-moult. The samples were either collected immediately at the time of moult and processed (Female A), collected the morning following a moult (Female B), collected immediately after moulting, preserved and stored at 4^{o}c and extracted the following morning (Female C) (Fig. 2.4). In these samples there were some peaks with retention times of 70-80 minutes that appeared unique to moulting females. One transient unique peak with a retention time of approximately 22 minutes was observed. If this was common to all females it could have represented a pheromone

candidate as there was no evidence of such a peak at this retention time in seawater samples that were either stored or collected several hours after the female moulted. However, this peak was not seen in other freshly collected seawater samples and it was concluded that these peaks were artefacts.

Due to the uncertainty of the half-life of the putative pheromone and the lack of a suitable whole animal bioassay by which to confirm odor tracking behavior, and hence the presence of a putative pheromone, other sample collection methods were adopted. These included extracts of the putative pheromone producing gland, the rosette glands, and collecting undiluted urine directly from the female.

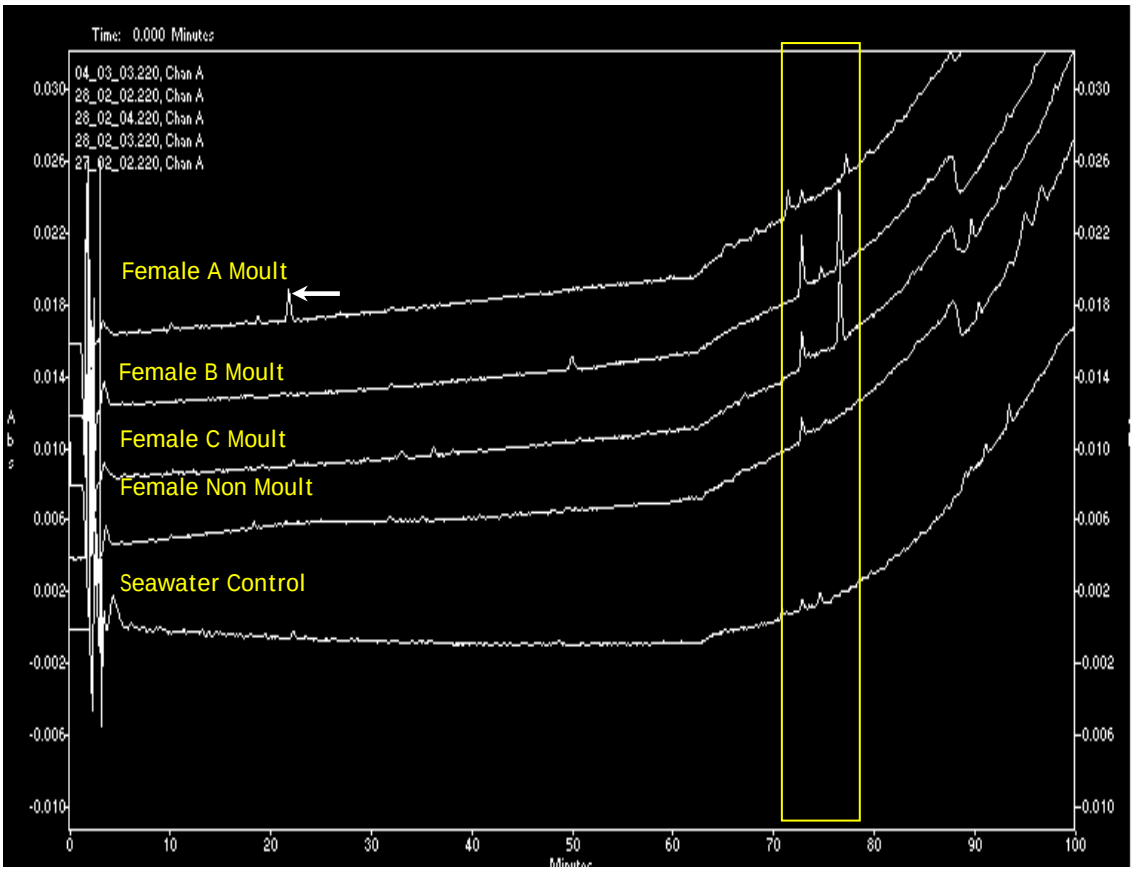


Figure 2.4. Reverse phase HPLC chromatograms of extracted compounds from moulted and non-moulted female *Penaeus semisulcatus* tank water. Highlighted region are peaks of interest (within box between retention time (RT) of 70-80 minutes) and at arrow. The peak at 21 minutes may be a degradable molecule.

ROSETTE GLANDS

Chromatographs of several tissue extracts, including the rosette glands demonstrated the presence of two to three major components. These had a retention time of between 17 and 22 minutes (Fig. 2.5). The primary component, and the first to elute, was common to all extracts and probably corresponds to general proteinaceous cytoplasmic compounds. The antennal gland and epithelium tissue also had a common component but there was evidence that this compound was also present, to a limited extent, to other tissue extracts. The chromatography of the antennal extract, assumed to be enriched in pheromone, did not reveal any component that was unique to females.

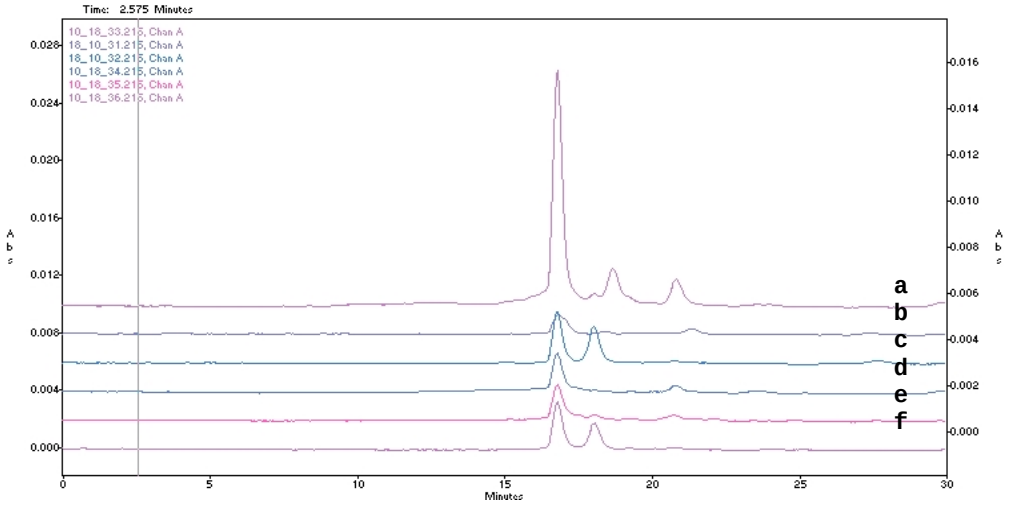


Figure 2.5. Chromatograph of female *Penaeus semisulcatus* tissue extracts filtered through a 5000 MW cut off filter. a: ovarian tissue; b: haemolymph; c: epithelial tissue; d: lymphoid organ; e: antennal gland; f: nephropore

URINE

The injection of fluorescein into the pericardial sinus allowed the visualisation of an urination event. Several minutes after a prawn was injected with fluorescein it was observed to urinate (Fig. 2.6). Although urination could be observed in this manner the prawn had to be restrained to allow photography (Fig. 2.7). It also had to be filmed under far blue and near UV wavelength and upon exposure to this lighting condition the prawn expressed a distinct escape response. It was concluded that this method was not appropriate to routinely monitor urination in prawns.

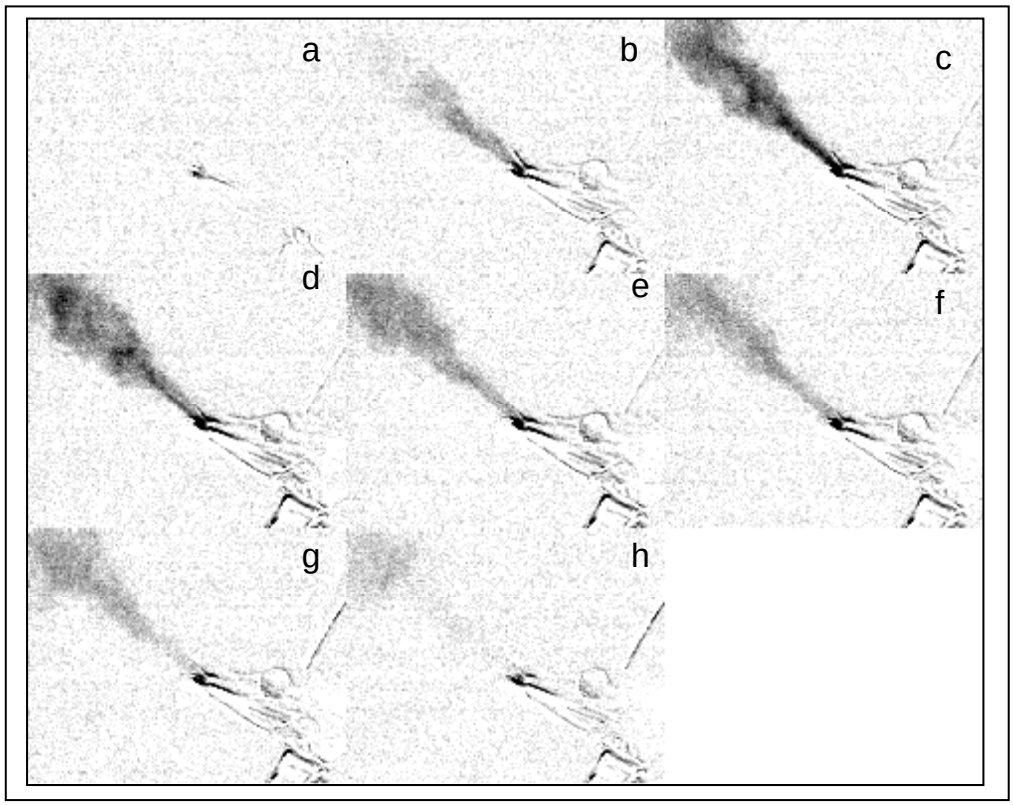


Figure 2.6. Photo-montage of fluorescein dye being released with urine from an adult, female *Penaeus semisulcatus*. Frame rate (start upper left hand corner a - h) for photographs was 1.5 frames per second.

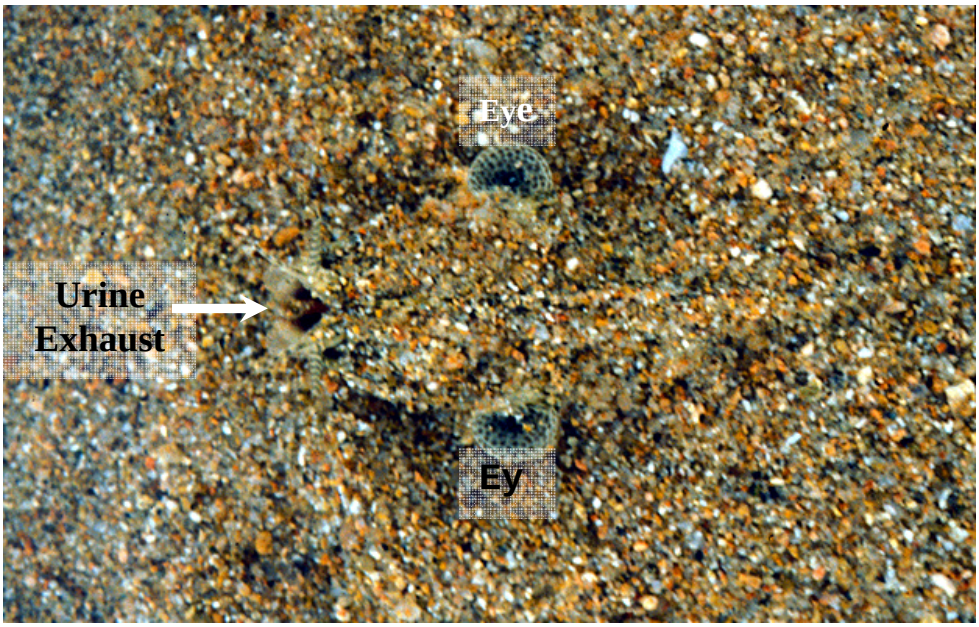


Figure 2.7. Probable 'nature' situation from which urine is released. Underwater photograph of female *Penaeus semisulcatus* buried in sandy bottom during daylight hours. Position of eyes labelled towards middle of photograph. A respiratory and urine exhaust 'tube' is formed by maxillipeds.

Injection of a single bolus of fluorescein followed by release of the prawn into a 50 L tank followed by monitoring of a sub-sample of the tank water demonstrated that it was possible to obtain data on the exact time of urination in unrestrained prawns (Fig. 2.8). It proved possible to monitor urination frequency from individual prawns over several hours (Fig. 2.9).

It was possible to detect the release of fluorescein from an individual prawns for several hours after injection (Table 2.3). Urination occurs in regular bouts with an average duration between events of 14 minutes.

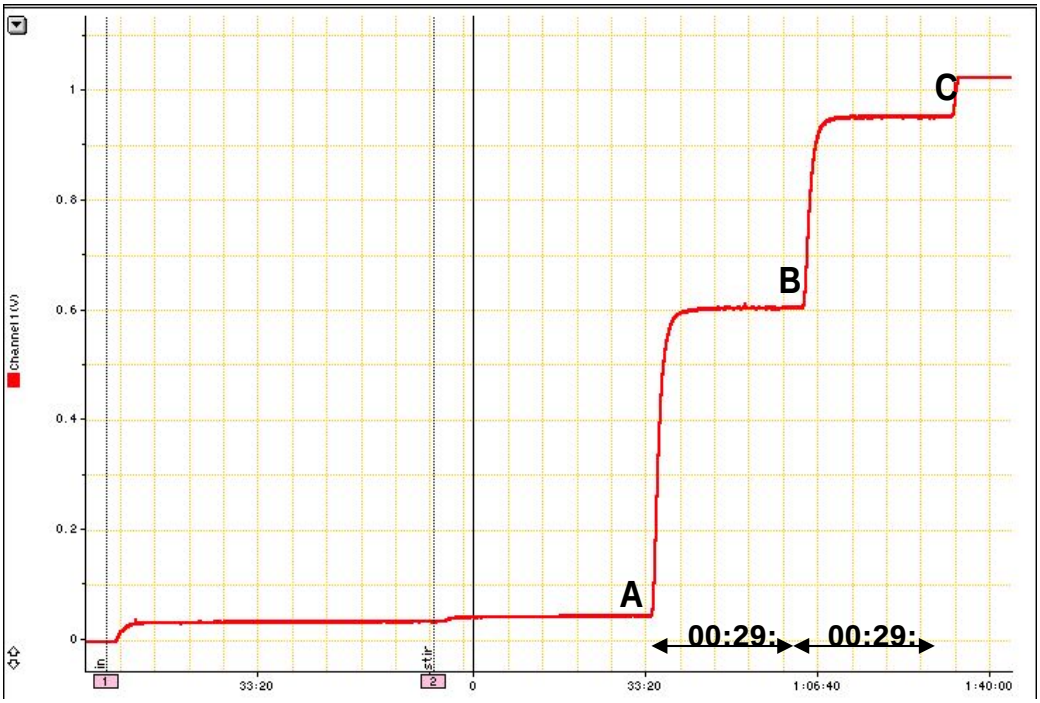


Figure 2.8. Urination frequency of a female *Penaeus semisulcatus*. Fluorescence units recorded in tank water after inoculation with 4µg/g bodyweight fluorescein. A, B and C represent urination events.

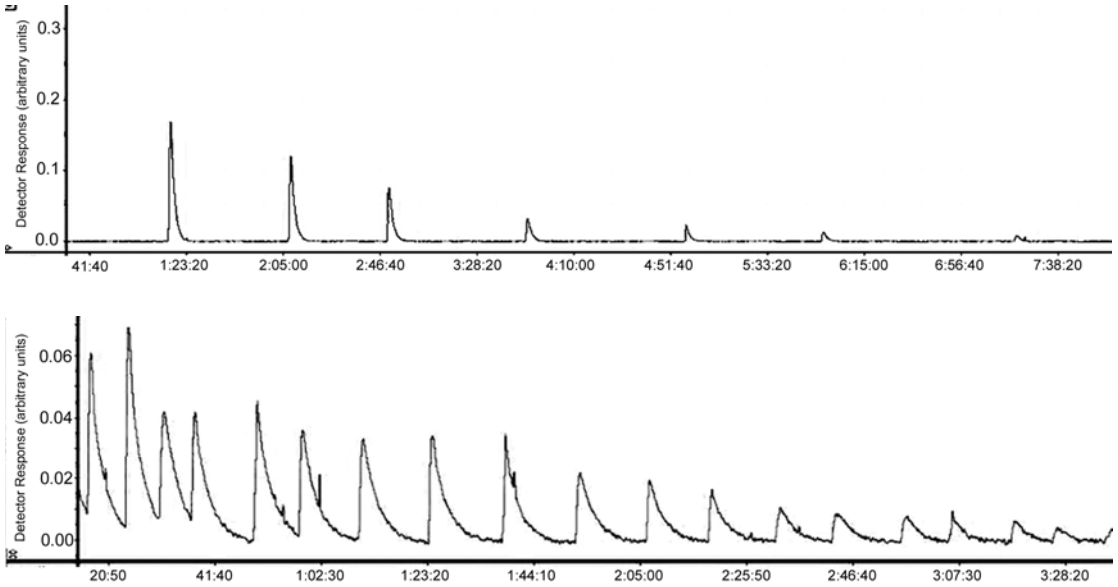


Figure 2.9. Urination frequency over several hours of two unrestrained female *Penaeus semisulcatus* after injection of Fluorescein determined by monitoring fluorescence in the aquarium water.

Table 2.3. Urination events of male and female *Penaeus semisulcatus* in various moult stages monitored over several hours.

Start Time	Sex	Stage	Urination Events	Duration Monitored (hrs)	Urinations /hour
08/25/2002 11:53 PM	Female	Moult	1	3.17	0.315789
08/26/2002 08:18 PM	Female	Moult	2	6.95	0.28777
08/26/2002 10:43 PM	Female	Moult	4	4.53	0.882353
08/14/2002 11:00 AM	Female	Post-Moult/Intermoult	2	5.83	0.342857
08/15/2002 09:11 AM	Female	Post-Moult/Intermoult	3	7.37	0.40724
08/16/2002 09:36 AM	Female	Post-Moult/Intermoult	5	6.97	0.717703
08/21/2002 08:45 AM	Female	Post-Moult/Intermoult	2	7.75	0.258065
08/22/2002 08:51 AM	Female	Post-Moult/Intermoult	9	8.93	1.007463
08/25/2002 05:56 PM	Female	Post-Moult/Intermoult	1	9.12	0.109689
08/26/2002 03:18 PM	Female	Post-Moult/Intermoult	2	7.35	0.272109
08/27/2002 03:02 PM	Female	Post-Moult/Intermoult	5	9.47	0.528169
08/28/2002 02:55 PM	Female	Post-Moult/Intermoult	1	9.57	0.10453
08/29/2002 11:41 AM	Female	Post-Moult/Intermoult	3	13.20	0.227273
08/30/2002 11:56 AM	Female	Post-Moult/Intermoult	3	4.92	0.610169
09/02/2002 08:55 AM	Female	Post-Moult/Intermoult	5	6.68	0.74813
08/19/2002 11:40 AM	Female	Pre-Moult	7	6.22	1.126005
08/27/2002 02:47 PM	Female	Pre-Moult	4	10.65	0.375587
08/28/2002 03:01 PM	Female	Pre-Moult	7	9.47	0.739437
08/29/2002 11:46 AM	Female	Pre-Moult	25	13.12	1.905972
09/02/2002 08:57 AM	Female	Pre-Moult	8	6.63	1.20603
08/12/2002 11:10 AM	Male	Post-Moult/Intermoult	4	3.82	1.048035
08/13/2002 10:14 AM	Male	Post-Moult/Intermoult	3	7.12	0.421546
08/14/2002 11:04 AM	Male	Post-Moult/Intermoult	6	5.78	1.037464
08/19/2002 11:36 AM	Male	Post-Moult/Intermoult	5	6.28	0.795756
08/21/2002 08:49 AM	Male	Post-Moult/Intermoult	4	7.67	0.521739
08/22/2002 08:56 AM	Male	Post-Moult/Intermoult	5	8.85	0.564972
08/30/2002 11:52 AM	Male	Post-Moult/Intermoult	18	4.98	3.61204

Time interval to urination was recorded from female prawns (N=20) grouped into three moult stages: recently moulted, post-moult/inter-moult and pre-moult (Fig. 2.10). Male prawns (N=7) were only recorded at post-moult/inter-moult (Fig. 2.10). The frequency of urination is similar across the groups with moulting and post moult inter-moult females generally showed the longest periods between urination events. It was difficult to obtain data on recently moulted females due to the high mortality associated with handling prawns with soft shells. As a result there are only a few data points for this moult stage. Males urinated more frequently than females but nothing is known of volume discharged per event.

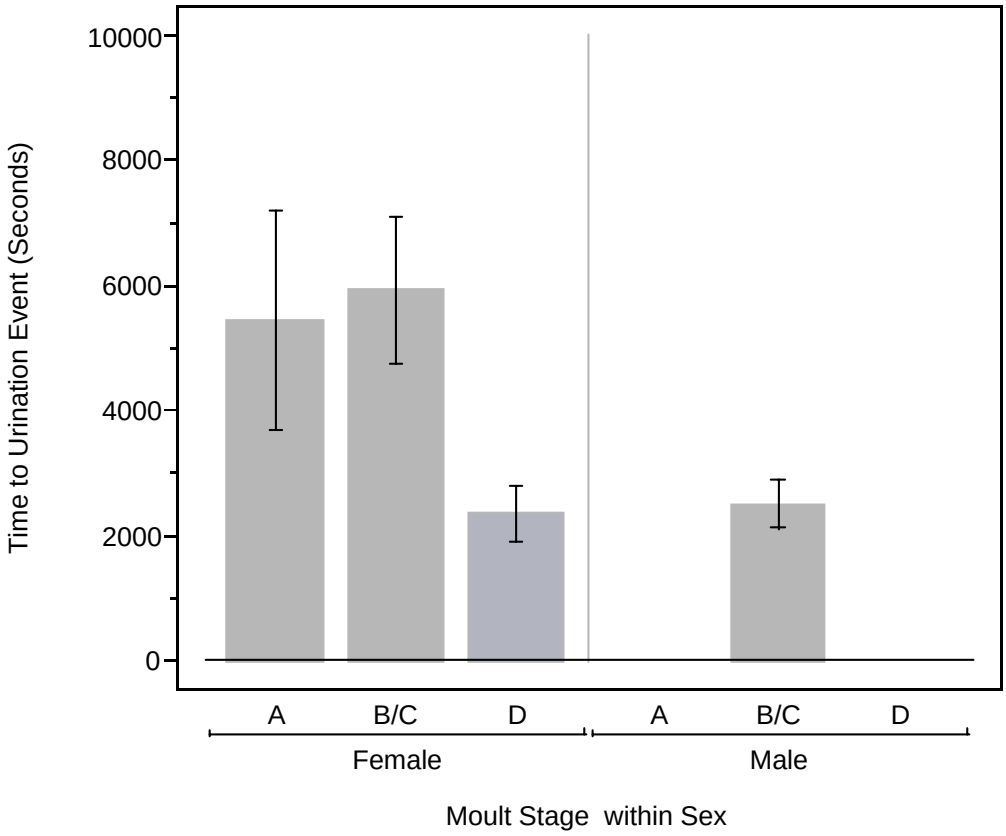
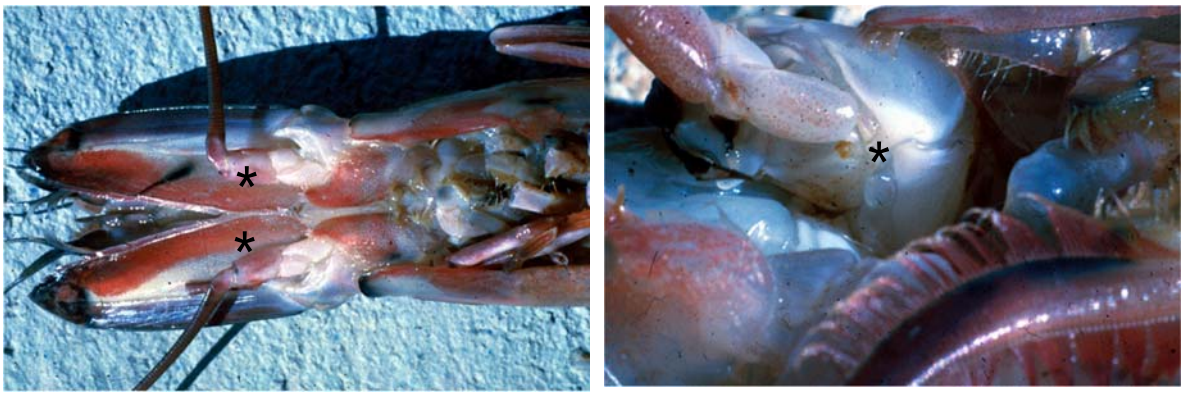


Figure 2.10. Interval between urinations of male and female *Penaeus semisulcatus* after injection of Fluorescein determined by monitoring fluorescence in the aquarium water. Prawns were grouped in three moult stages: recently moulted (A), post-moult/inter-moult (B/C) and immediate pre-moult (D).

As an alternative to the collection of diluted urine from seawater, direct urine collection was investigated by catheter. The nephropore is located on the ventral side at the base of the antennules and appears as a small nib of fleshy epidermis. If the maxillipeds are dissected away the nephropore is readily seen (Fig. 2.11).



(a) **(b)**

Figure 2.11. Ventral side of prawn showing an overall view of the location of the nephropore (*) on the dorsal medial plate of the basal stalk of the antennae (a) and after removal of the maxillipeds (b).

Histological analysis of putative nephopore confirmed that the fleshy bud at the base of the antennule was indeed the orifice from the antennal gland covered by a flap of epidermis and that it was possible to gain access to it with a catheter (Fig. 2.12).

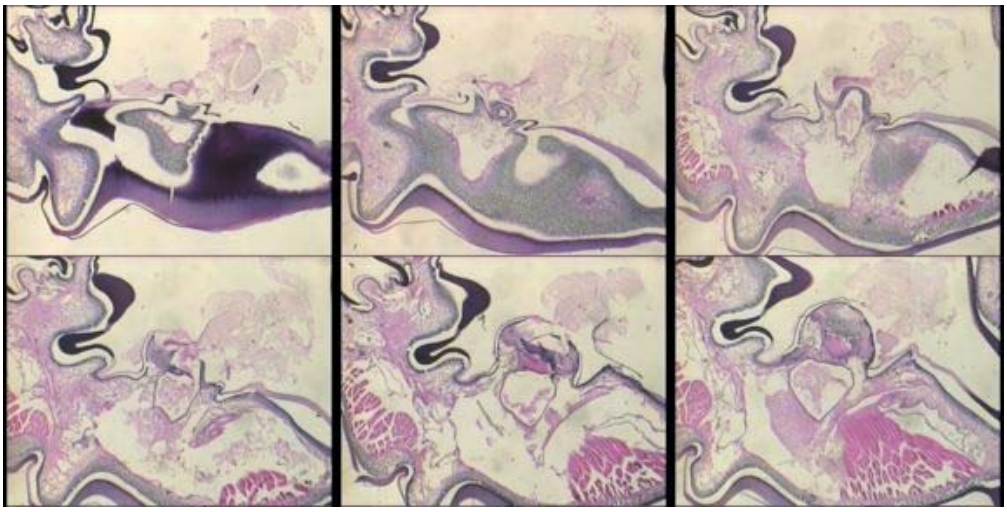


Figure 2.12. Serial section through the nephropore of *Penaeus semisulcatus*. The 'valve' opening (a) allows a cannula to be inserted and a sample of urine obtained directly from the antennal gland.

Fluorescein was concentrated in the urine compared to the haemolymph (Fig. 2.13). The collection of urine by gentle squeezing of the base of the antennules appears to run the risk of contaminating the fluid with water from the gill brachial chamber, as the fluorescein levels of putative urine collected in this way are equivalent to seawater itself. Measurement of ammonia and nitrate indicates that either catheter collected urine or that expressed by squeezing gives equivalent samples (Fig. 2.14).

However, based on the two collection methods it appears that collection by catheter would ensure that a true urine sample is collected.

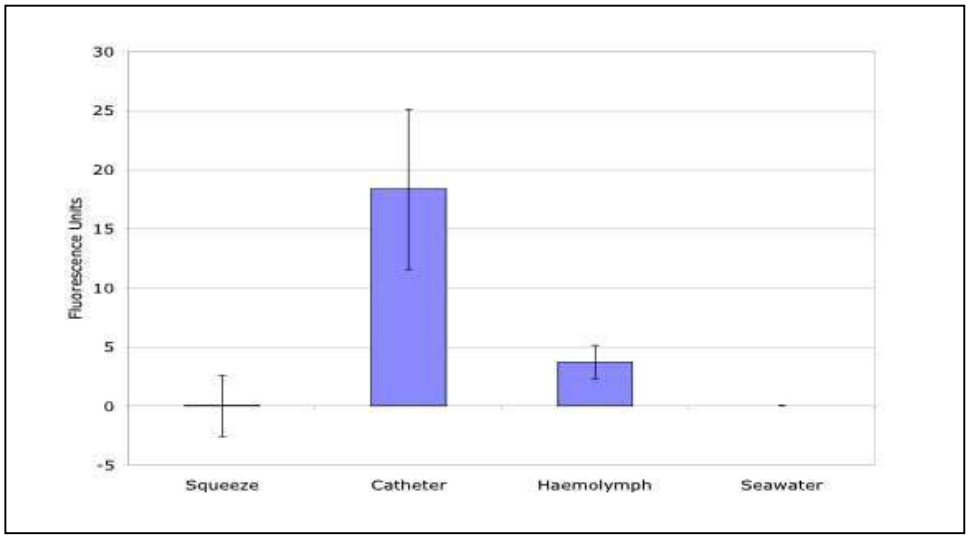


Figure 2.13. Concentrations of fluorescein present in *Penaeus semisulcatus* following an injection of fluorescein into the pericardial sinus (n=5).

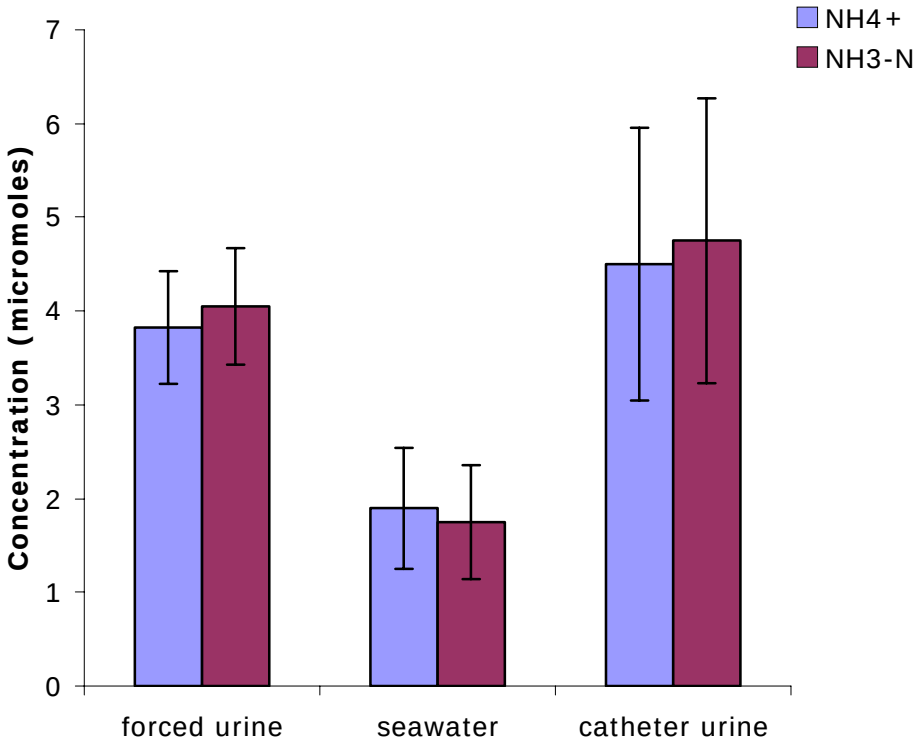


Figure 2.14. Concentrations of ammonia and nitrate in body fluids from *Penaeus semisulcatus* to confirm that collection of urine is differentiated from other body fluids (n=5).

PHEROMONE PURIFICATION FROM URINE

Size exclusion chromatography of urine samples from revealed several peaks that were sufficiently concentrated to process on a mass spectrometer (Fig. 2.15). Based on size exclusion chromatography alone it was not possible to clearly identify any peaks that might have been specific to moulting females.

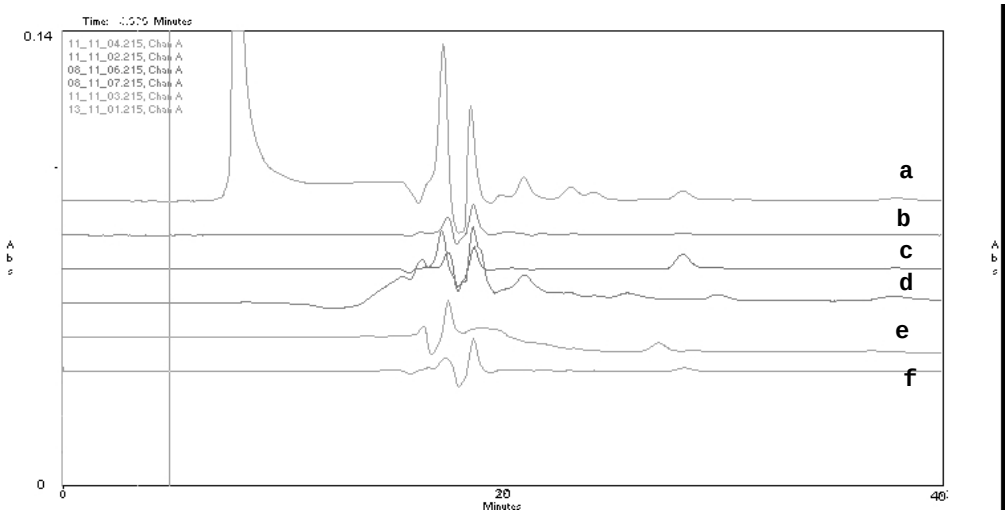


Figure 2.15. Chromatographs of urine samples (1:10 dilution) from *Penaeus semisulcatus* and *P.monodon* passed through a Superdex Peptide column in 2% Formic Acid in Milli-Q Water at 1 mL/min and detected by UV at 215 nm. a: *P.semisulcatus* Antennal Gland; b: *P.semisulcatus* female urine; c: *P.monodon* female urine; d: *P.semisulcatus* female urine; e: *P.monodon* female urine; f: *P.semisulcatus* male urine.

For mass spectrometry analysis the prawns were grouped according to sex and into recently moulted, post-moult and inter-moult individuals and the resulting signals from these compared (Fig. 2.16). Two specific compounds to female prawns of m/z 316.0 and 344.1 were found. These compounds were singly charged and in the mass range of small peptides. A compound of m/z of 344.1 was also present, at a lower concentration, in a female that was entering inter-moult (Figs. 2.17 and 2.18).

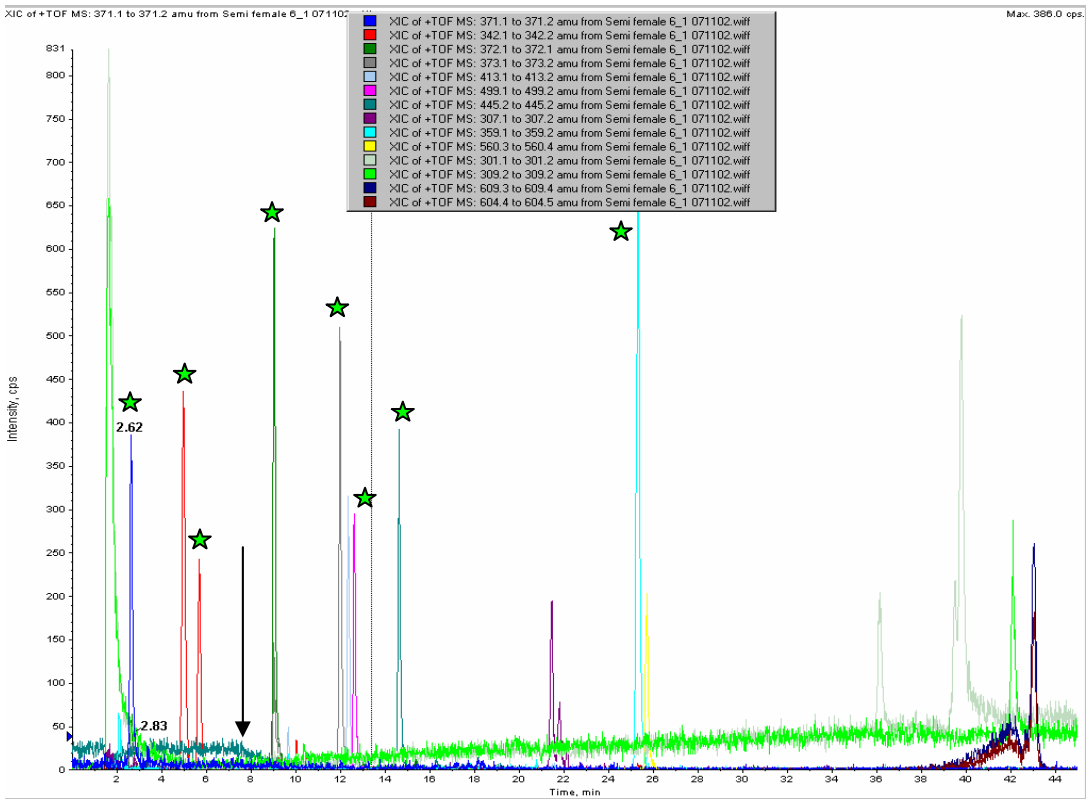


Figure 2.16. LC/MS chromatograph of female *Penaeus semisulcatus* urine. Colouration distinguishes peaks of different mass over time. C-18 HPLC column 150x2.1mm Phenomenex 300A Flow rate = 300 uL/min. 0.1% formic acid (A) / ACN (B).

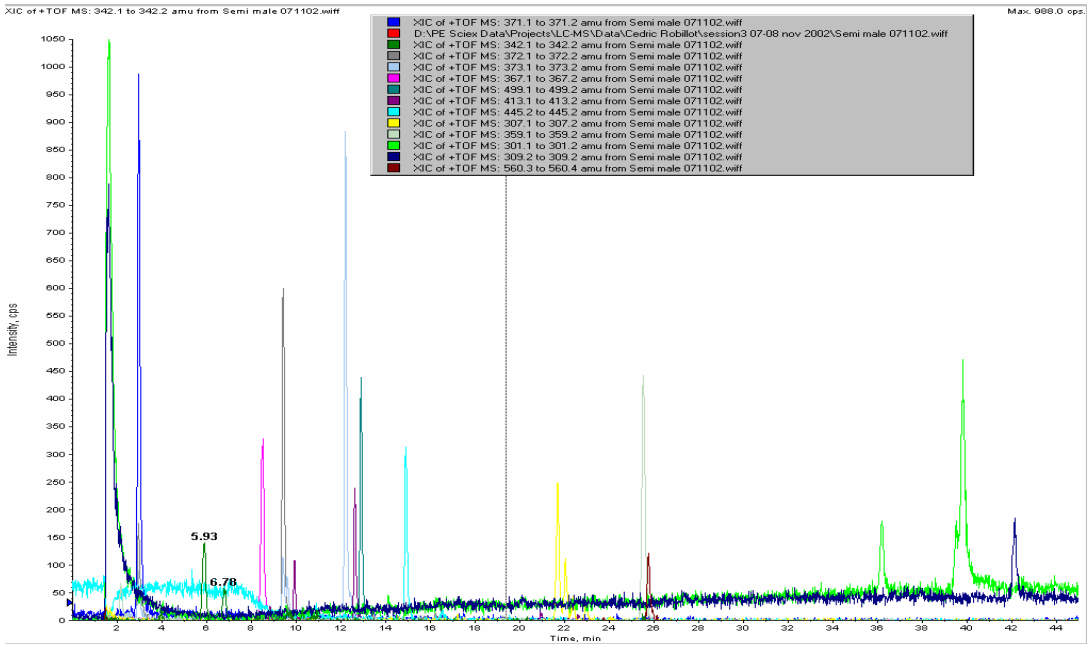


Figure 2.17. LC/MS chromatograph of male *Penaeus semisulcatus* urine. Colouration distinguishes peaks of different mass over time. RP-HPLC column 150x2.1mm Phenomenex 300A Flow rate = 300 uL/min. 0.1% formic acid (A) / ACN (B).

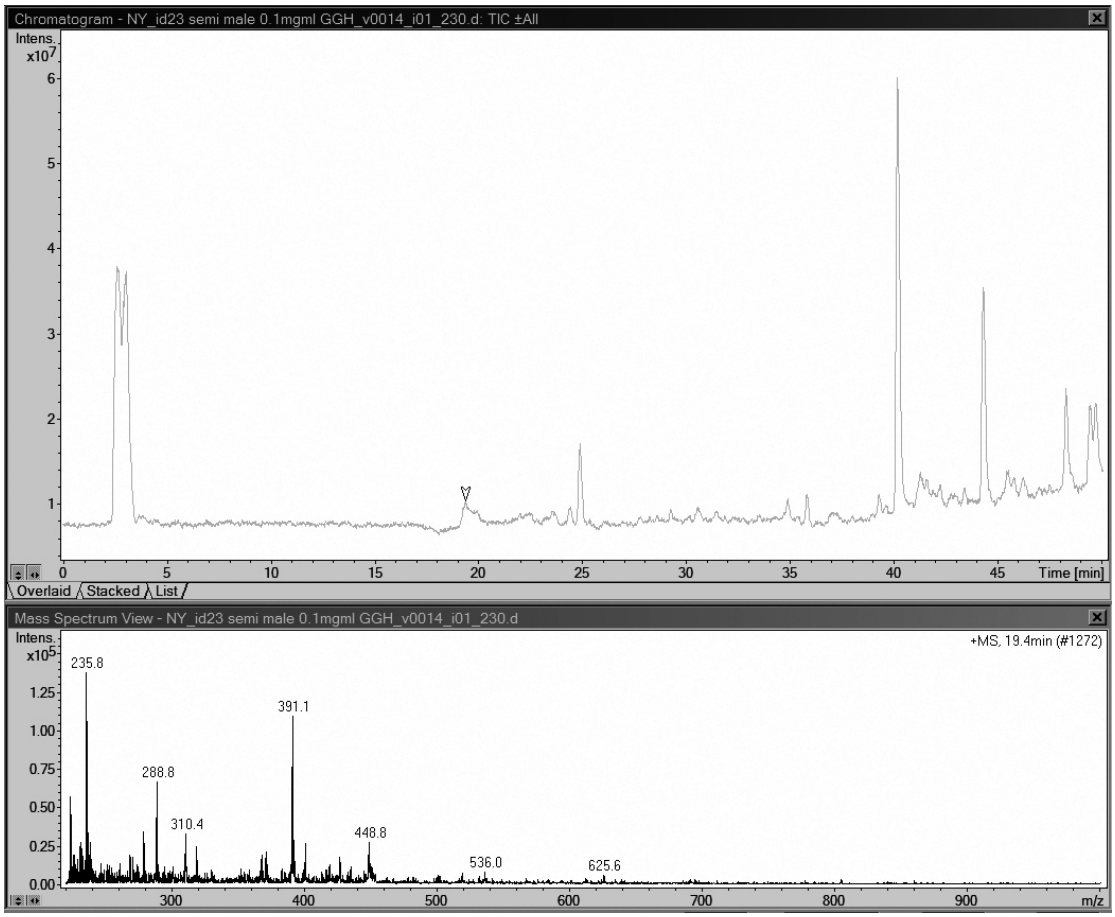


Figure 2.18. Example of typical LCMS analysis of urine showing total ion chromatograms. Peaks were selected (arrow – upper figure) from the LC trace and analyzed between 220 and 1,000 m/z. Base peak chromatograms were generated for individual m/z and peak ion intensity and retention time recorded.

LC/MS highlighted a number of peptides in urine samples from female *Penaues semisulcatus* that were unique and not found in males. Differences and similarities in urine from male and female prawns in various moult stages were examined (Tables 2.4 and 2.5). Approximately 11 signals were common in both male and female urine samples. This urine "fingerprint" provided a baseline for investigating moult stage and sex differences. Of 133 female-specific compounds in 9 females examined, 23 of these were present in 3 or more individuals. In comparison only 15 male-specific compounds in 4 males examined were found. Of these only 1 compound was specific to in more than one male and only common to two of them. If these are reproductive pheromone-like compounds then the presence of a larger array of compounds in female urine supports the physiological evidence collected regarding increased male olfactory abilities.

Table 2.4. Number of molecules purified, but not identified nor characterized, from male and female *Penaeus semisulcatus* prawns either in inter-moult or immediately post-moult.

	INTER-MOULT PRAWNS		POST-MOULT PRAWNS	
	Number of sex-specific molecules	Molecules m/z range	Number of sex-specific molecules	Molecules m/z range
Female	133	240 to 458	23	263 to 497
Male	15	230.8 to 929.6	5	243.8 to 522.9

Table 2.5. Mass/charge ratios of unique molecules purified, but not identified nor characterized, from male and female *Penaeus semisulcatus* prawns either in inter-moult or immediately post-moult.

Moulted female (m/z)	**Moulted male (m/z)**	**Male specific (m/z)**	**Female specific (m/z)**	**Female specific (cont.) (m/z)**	**Female specific (cont.) (m/z)**	**Female specific (cont.) (m/z)**	**Female specific (cont.) (m/z)**
263	243.8	240	230.8	285.8	326	392.5	496.1
269.1	292.9	240.9	232	287.8	330	394.1	497
277.5	295.9	252.9	235.8	288	331	396	516.1
290.8	480.9	272	236.9	289	334	396.1	546.1
294.8	522.9	290.7	237.9	290.4	336	408	560.1
310.5		306.9	238.8	290.8	342	409.6	576.1
316		307	242.9	291.8	343.1	410	614.6
316.2		314.1	243	291.9	344.1	417.1	630.8
326		324.8	244.9	292.1	345.5	419.1	649.2
344.1		325	245	292.6	347	422.2	672.5
362.1		345.9	255.4	293.8	350.1	423.1	738.7
368.1		356.9	256.6	294	358	427	747.3
372.1		359.9	257.9	294.7	358.2	427.1	871.4
376.1		426.2	258.7	294.8	359.1	427.9	929.6
377		458	259.8	297	361	431.2	
386.8			260	298.8	362	435.1	
396			262.9	300.9	362.1	438.9	
438.9			263	304	367	439.2	
448.7			264.9	306.1	367.1	447.1	
448.8			265.7	309	368.1	448.7	
459			269.1	310.5	372.1	448.8	
479			269.4	313.9	373.1	457.9	
497			270.7	316	376	459	
			274.8	316.2	376.1	459.2	
			276	316.9	377	461.1	
			277.5	318.7	379	478.1	
			278	318.9	380	479	
			278.9	320.1	381.9	482.2	
			281.9	322.1	384	485	
				325.9	386.8	489.1	

Discussion

The purification of a putative reproductive pheromone was severely curtailed by the lack of a suitable bioassay in order to indicate when a sample of seawater should be collected. Penaeid prawns have many characteristics making them inappropriate as a bioassay model species by which to identify the presence of a reproductive pheromone. The in-line chromatography approach, while valid and efficient if it could be backed up with whole animal bioassay data, was based on little more than educated guesswork on when a sample should be collected. It is perhaps therefore not surprising that a chemical signal could not be detected or purified using the in-line chromatography approach. The collection of samples for batch chromatography suffered from similar restrictions. Based on known mate searching behavior of other crustacea it was assumed that a putative reproductive pheromone would be released by a female penaeid prawn late in the day before or on the night of moulting. However, samples collected at this time and examined chromatographically also did not reveal any readily identifiable chemical candidate.

Most published evidence on crustacean pheromone indicates that the primary source is from the urine (see introduction). As previously reported, histological examination has shown secretory ducts emptying into the antennal gland, the main excretory organ of crustacea. Urine is briefly stored in a bladder and leaves the body through the nephropore. Pheromone may be fed into the antennal gland as secretory products from the rosette glands that line the bladder. The collection of urine, if made at the appropriate time in the reproductive cycle, should contain active fully processed pheromone. It might have been expected that there would be an increase in urination around the time of the moult in females coinciding with signalling to male prawns. However, female urination pattern did not exhibit an increase at the time of moulting. Indeed, immediately moulted female prawns showed longer periods between urination events. As it is assumed that there is only a short period of receptivity in penaeid prawns we would expect that females would be signalling rapidly to attract a mate. However, urination frequency does not offer any information on whether any one bout of urination contains a pheromone.

Collection of point samples of urine by catheter from females approaching moult did not reveal any chemical signal with the reverse phase chromatography and detection methods we used. However, although there is no penaeid prawn bioassay to confirm biological activity, we were able to purify 23 female specific compounds by size exclusion chromatography and mass spectrometry. However, we were unable to characterise these further as a sample of several orders of concentration greater than that obtained from the urine is required and hence they remain uncharacterised.

A contributing factor to the difficulties in chemically identifying the putative reproductive pheromones may be due to their low concentration even in urine. For example, molluscs are capable of detecting, and responding to, predator odors or pheromones at concentrations as low as 10-8 to 10-11 M. In the blue crab (*Callinectes sapidus* odor trail searching by extracts of clams (*Mercenaria*) is stimulated to concentrations as low as 10-13 to 10-18 g dry clam/L (Pearson and Olla 1977). Assuming equivalent pheromone detection ability in a prawn and that a pheromone could be released at a concentration of 10-2 M in 10 uL of urine into a volume of 20 L of seawater, i.e the volume that females were held in for the bioassay work, then there would be 0.5 x 10-8 M of pheromone present in the whole tank. Although this concentration should be detectable by the HPLC methodology used in this study we were unable to convincingly isolate it. However, the mass spectrometer data indicates that there are a number of possible chemical candidates that are unique to females. Following on from the above assumption, in the wild the pheromone would disperse over time and would be present at a concentration of 10-11 M in 10,000 L of water. This volume of water could be expressed as a plume 1000 m long x 1 m wide x 10 cm thick. Hence an original release of 10 µL of urine at a concentration of 10-2 M could generate an chemo-attractant plume 1 km long and still be within a concentration of 10-11 M. This concentration is known to be detectable by other marine organisms such as molluscs. In ovigerous mud crabs, a threshold concentration of a pheromone tripeptide as low as 10-21 M was capable of inducing a response (Decho et al. 1998). If penaeid prawns respond in a similar manner, then the 'chemo-attractant area' of a released pheromone would be more than sufficient to concentrate prawns from a significant distance.

A recent alternative that is now available for identifying molecular candidates as pheromones is the use of a molecular biological approach. The significance of olfaction in eurkayotes is demonstrated by the proportion of the entire genome devoted to olfaction, with as much as 4% of it encoding for proteins associated with smell (Firestein 2001). In mammals, the chemoreceptors are encoded by the largest family of genes known to exist. For example, mice, which have a highly develop sense of smell, have approximately 1,2000 genes for olfactory receptors (Li et al. 2004). Few invertebrate genomes have been completely sequenced, but in the nematode (*Caenorhabditis elegans*) it is known that over 500 genes encode for chemoreceptors, which are mainly encoded for within the chemoreceptor neurons themselves (Bargmann 1998). In vertebrates an individual chemosensory cell is believed to have only one or a small number of odorant receptors whereas in invertebrates, at least in *C. elegans*, multiple receptor genes are expressed in one chemosensory cell (Troemel et al. 1997, Malnic et al. 1999). Genomic studies in *C. elegans* have demonstrated that the behavioural response to an odor is not defined by the receptor itself, as the expression of a chemosensory gene from one cell in another one can change a behavioural from attraction to repulsion (Troemel et al.

1997). Although invertebrate chemosensory receptors have widely divergent sequences, most share a common structure containing seven transmembrane domains (Krieger and Breer 1999). This has allowed several homology-based approaches from genome databases, including gene sequence searches for seven transmembrane domain receptors and cloning strategies to identify rare messenger RNA expressed only in olfactory neurons (Clyne et al. 1999, Vosshall et al. 1999). More recently a technique to scan genome databases for particular structures, rather than sequences, with specific computer algorithms that identifies open reading frames (ORFs) from DNA databases based on five variables, has allowed the identification of large families of odor receptor genes (Warr et al. 2001). Although this is achievable for those organisms whose genomes have been completely sequenced, such as *Drosophila*, by exploiting the commonality of chemoreceptor genetics, such as the conserved seven transmembrane domains, it offers one approach by which to analyze the olfactory system of prawns and to identify the molecular nature of the pheromone that can bind to the chemoreceptors. This approach is independent of a bioassay directed purification strategy and may be the only approach open for the identification of the molecular nature of penaeid prawn pheromones.

OBJECTIVE 3
To identify a mechanism for manufacturing a bait incorporating these novel attractants

Introduction

An increase in searching activity in response to a dissolved substance from a moulting female was found as well as odor tracking to a chemo-attractant from a food source (Objective 1). Although a number of molecular candidates as putative female pheromones were found by mass spectrometry analysis of urine in Objective 2 they could not be chemically characterised and therefore could not be tested as a pheromone bait in a pot trap. The project procured manufactured pot traps from the north Pacific shrimp fishery and the design of the trap typically used in the north Atlantic shrimp fishery. Rather than using a pheromone bait we used food as bait for the traps. The objective was to determine if these trap designs were useful to pot trap penaeid prawns using the chemo-attractants of food that penaeid prawns could odor track to its source.

Methods

The ultimate goal of this line of enquiry was to examine the possibility of developing pot traps as an alternative harvest technology for penaeid prawns. We tested 3 pot trap designs that are used in the north Atlantic north and Pacific pot trap commercial fisheries. One pot trap was a replicate of the design that is used in the north Atlantic shrimp fisheries of Maine, USA. The pot is typically made of galvanised steel mesh (as used for this study) but occasional from vinyl coated mesh. The only entrance to the pot is from the top and is through a rectangular V-shaped opening. This type of pot is typically set in areas that cannot be trawled due to their topology as the areas are steeply sloped and represent ancient river beds when sea levels were greatly lower than today but now form part of the continental shelf. The other two types were from pot trap manufacturers in Vancouver, Canada and were designed to capture cold water pandalid shrimp. They had a steel frame with a nylon mesh covering. One design was a small folding rectangular trap used in the recreational and commercial fishery. There are two entrances at either end of the trap. A shrimp gains access by entering a sloping mesh, slightly raised from the base of the pot, and through a small circular entrance. The other trap is circular and has 3 entrances. Access is made as in the other pot design but the sloping mesh is further raised from the base of the pot.

The bioassay tank previously described (see whole animal bioassay methods) was modified (channels removed). Two females and 6 male prawns (*P.semisulcatus*)

were placed into the tank in the morning and allowed to bury in the sand prior to the start of the trial. The tank was provided with fresh seawater at the opposite side of the tank from the outlet so that water circulated in the tank over the course of the experiment. Water was added at approximately 30 L/min and temperature was between 28 and 29°C over the course of the trials. Pot traps were placed into the arena at 6pm and filming began at 7pm and went through the night till 0400 hours.

The position of the animals in the tank in relation to the traps as well as the number of animals trapped over the course of the night was monitored. Filming methods were the same as previously described for the whole animal bioassay. Footage was collected at 1 frame per second and a frame size of 384x288 pixels. For analysis the footage was divided into 1 hour segments of 3600 frames each. The images were loaded into image analysis software, Image J (Wayne Rasband NIH). Each frame was subtracted from a background image created from an average of all frames over an hour. In effect this removed the background but left the moving animals in the tank. The image was then threshold leaving only the prawns in the image. The location of the pixels that represented each prawn was recorded for each frame. With the difficulties previously mentioned regarding tracking the prawns we looked at overall patterns and positions of all animals in the tank. All scatter plots and density plots were generated using JMP statistical software. Scatter plots showed the individual points of the prawns over a 3 hour period. Bivariate density estimation modelled how dense the data points were at each point in that surface. The plot adds a set of contour lines showing the density. This plot results in 5% of the points being below the lowest contour, 10% below the next contour, and so forth. The highest contour has about 95% of the points below it.

To determine whether prawns were spending time around the traps we generated a border around the traps and recorded the number of times prawns entered the region around the trap over the 1 hour periods.

Results

In all tests nocturnal activity of prawns was intense. Based on the scatterplot of prawn movements and density profiles taken when food was placed in the tank with no trap there was a definite increase in movement around the food source (Fig. 3.1a). However, the general activity of the prawns was to spend the majority of time around the borders of the tank tracing the walls back and forth similar to what we saw with the whole animal bioassay trials (Objective 1). Nevertheless, based on the contoured plot prawns did spend time in the immediate vicinity of the food source.

Of the 3 types of pot traps tested, with or without food, the patterns were fairly similar (Figs. 3.1b,c,d,e,f). However, the round rope trap was more attractive to the prawns with more time spent within the area surrounding it, which is visible in the density profiles (Fig.3.1f) and histogram (Fig. 3.2). Overall however no prawns were seen to enter the traps or found within the traps the following morning (Table 3.1).

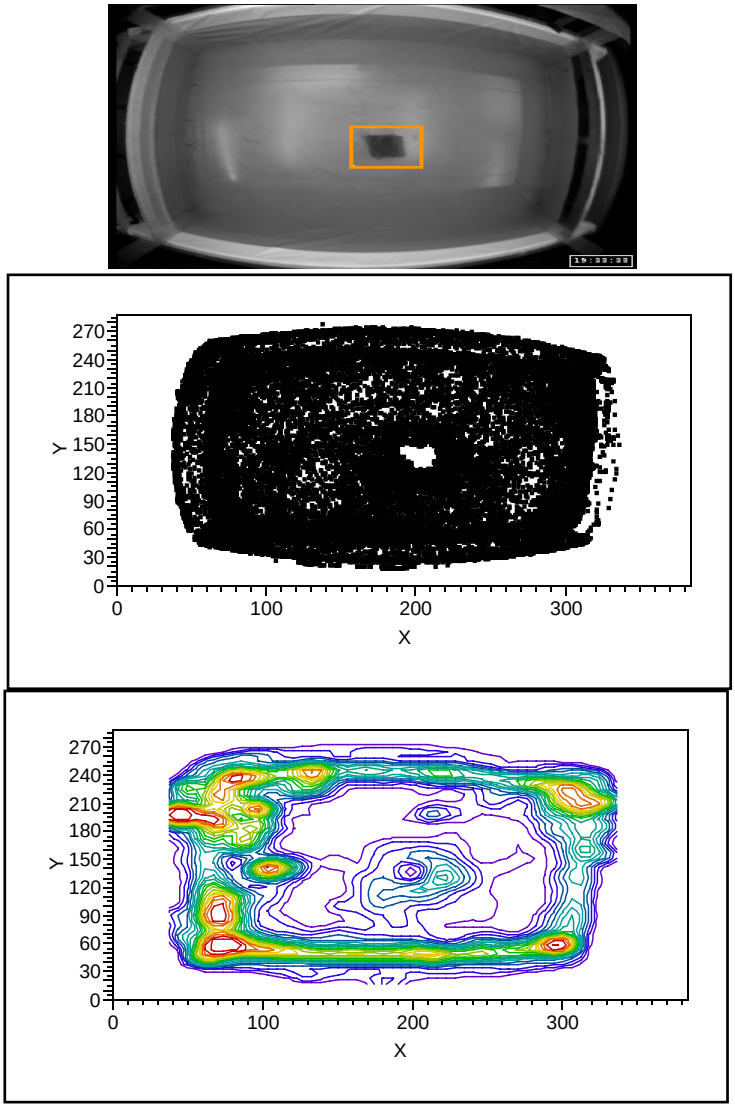


Figure 3.1a. Activity of prawns in the arena tank without a pot trap but in the presence of a mesh bag containing 2 green lipped mussels (*Perna canaliculus*) fixed in place as a positive control. Analysis of 2 female and 5 male prawns (*P.semisulcatus)*. Photograph of arena tank and mesh bag, highlighted in a superimposed rectangle on the image (a). Position of prawns (b) and 'hot spots' of activity (c) in tank over a 3 hour period (1900 hrs to 2200 hrs) following placing bait into tank.

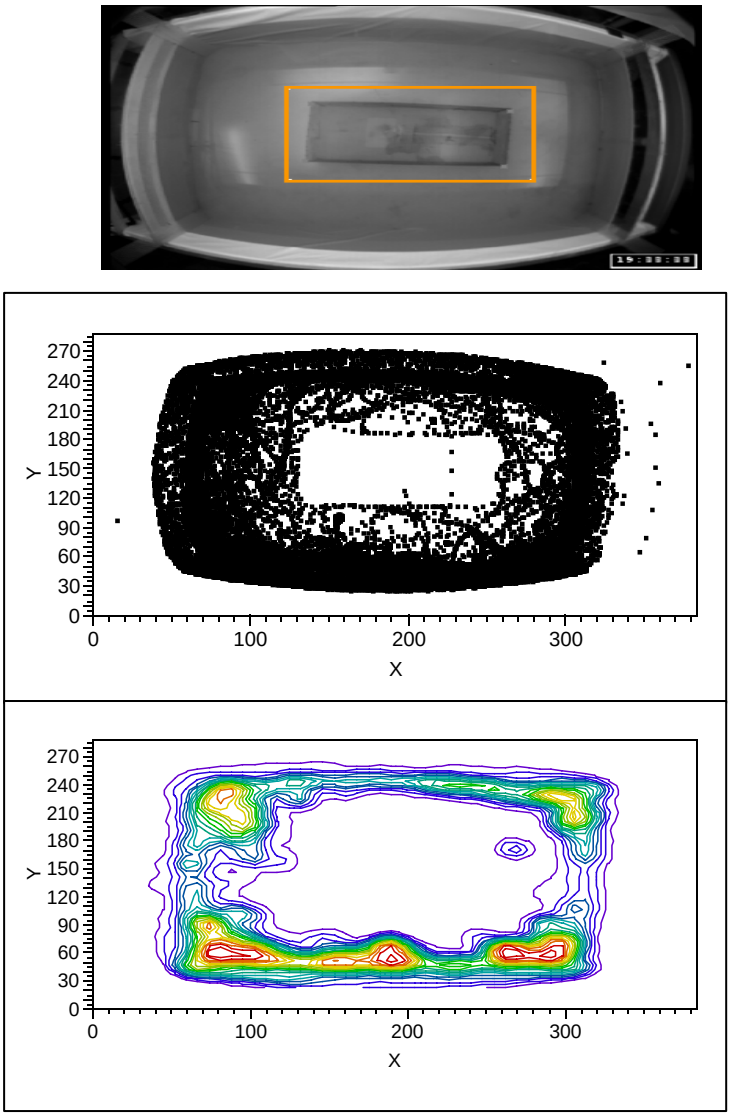


Figure 3.1b. Activity of prawns in the arena tank with the north Atlantic shrimp pot trap design with no food, i.e. empty pot, as a negative control. Analysis of 2 female and 5 male prawns (*P.semisulcatus)*. Photograph of arena tank and mesh bag, highlighted in a superimposed rectangle on the image (a). Position of prawns (b) and 'hot spots' of activity (c) in tank over a 3 hour period (1900 hrs to 2200 hrs) following placing trap into tank.

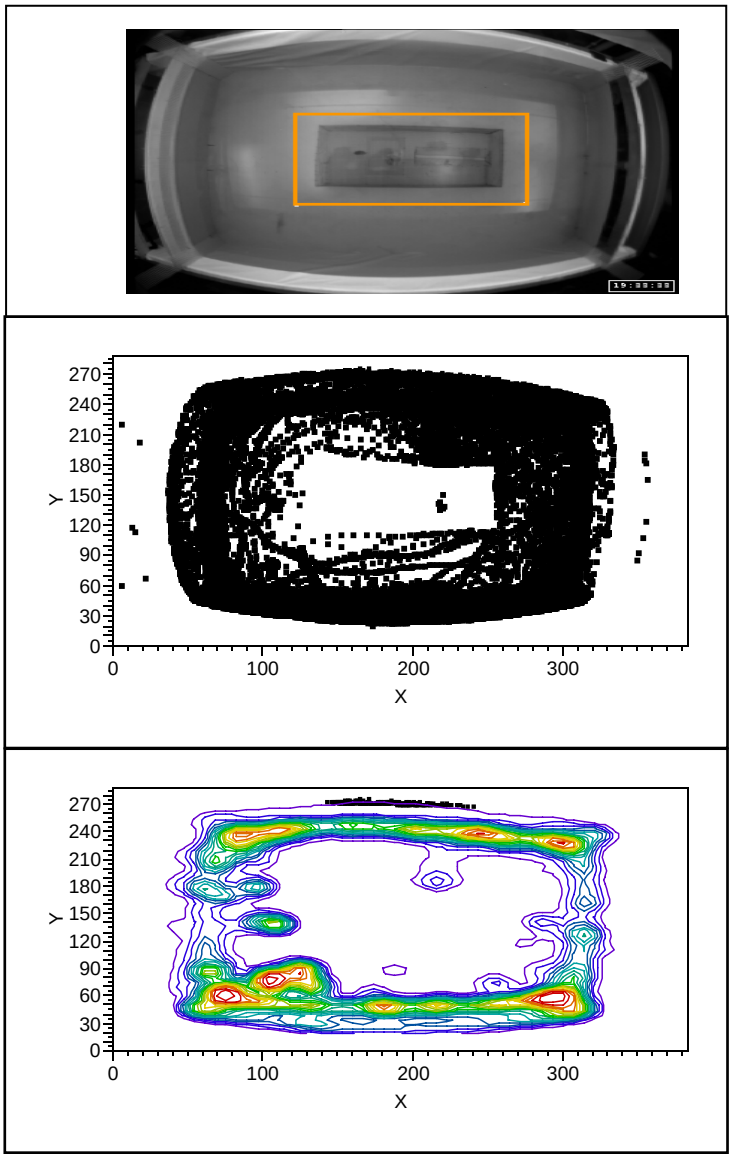


Figure 3.1c. Activity of prawns in the arena tank with the north Atlantic shrimp pot trap design baited with 2 green lipped mussels (*Perna canaliculus*). Analysis of 2 female and 5 male prawns (*P.semisulcatus).* Photograph of arena tank and mesh bag, highlighted in a superimposed rectangle on the image (a). Position of prawns (b) and 'hot spots' of activity (c) in tank over a 3 hour period (1900 hrs to 2200 hrs) following placing bait into tank.

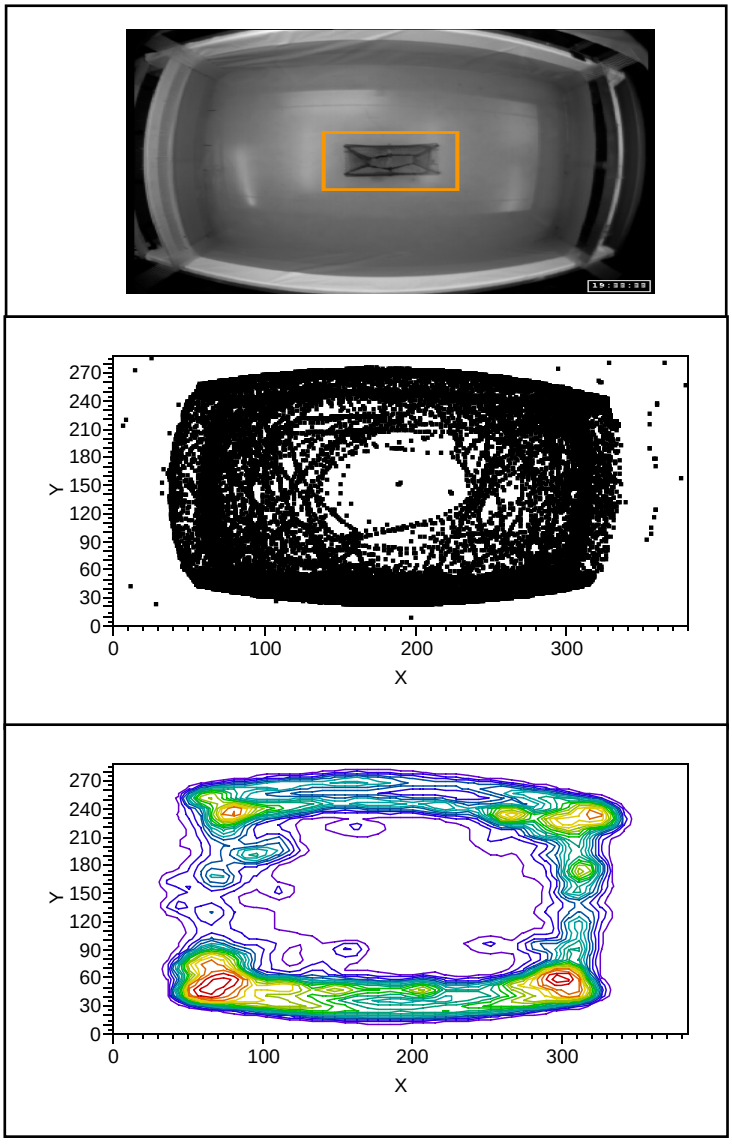


Figure 3.1d. Activity of prawns in the arena tank with the north Pacific small shrimp pot trap design with no food, i.e. empty pot. Analysis of 2 female and 5 male prawns (*P.semisulcatus).* Photograph of arena tank and mesh bag, highlighted in a superimposed rectangle on the image (a). Position of prawns (b) and 'hot spots' of activity (c) in tank over a 3 hour period (1900 hrs to 2200 hrs) following placing bait into tank.

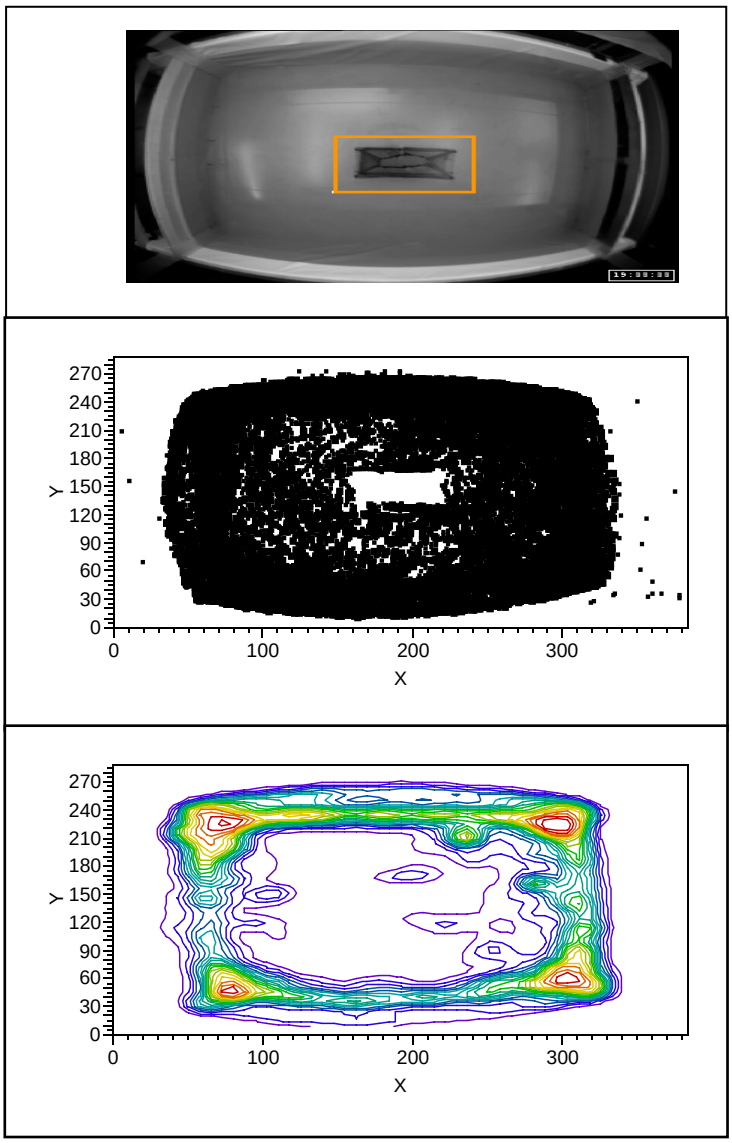


Figure 3.1e. Activity of prawns in the arena tank with the north Pacific small shrimp pot trap design baited with 2 green lipped mussels (*Perna canaliculus*). Analysis of 2 female and 5 male prawns (*P.semisulcatus).* Photograph of arena tank and mesh bag, highlighted in a superimposed rectangle on the image (a). Position of prawns (b) and 'hot spots' of activity (c) in tank over a 3 hour period (1900 hrs to 2200 hrs) following placing bait into tank.

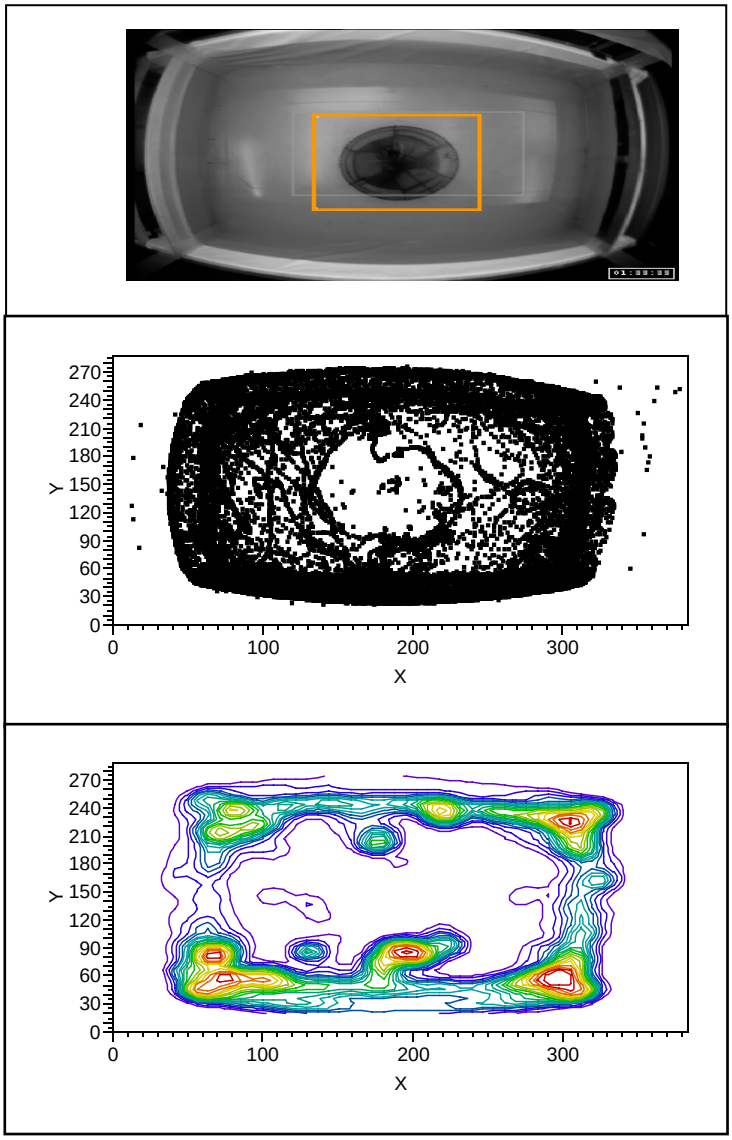


Figure 3.1f. Activity of prawns in the arena tank with the north Pacific large circular shrimp pot trap design baited with 2 green lipped mussels (*Perna canaliculus*). Analysis of 2 female and 5 male prawns (*P.semisulcatus)*. Photograph of arena tank and mesh bag, highlighted in a superimposed rectangle on the image (a). Position of prawns (b) and 'hot spots' of activity (c) in tank over a 3 hour period (1900 hrs to 2200 hrs) following placing bait into tank.

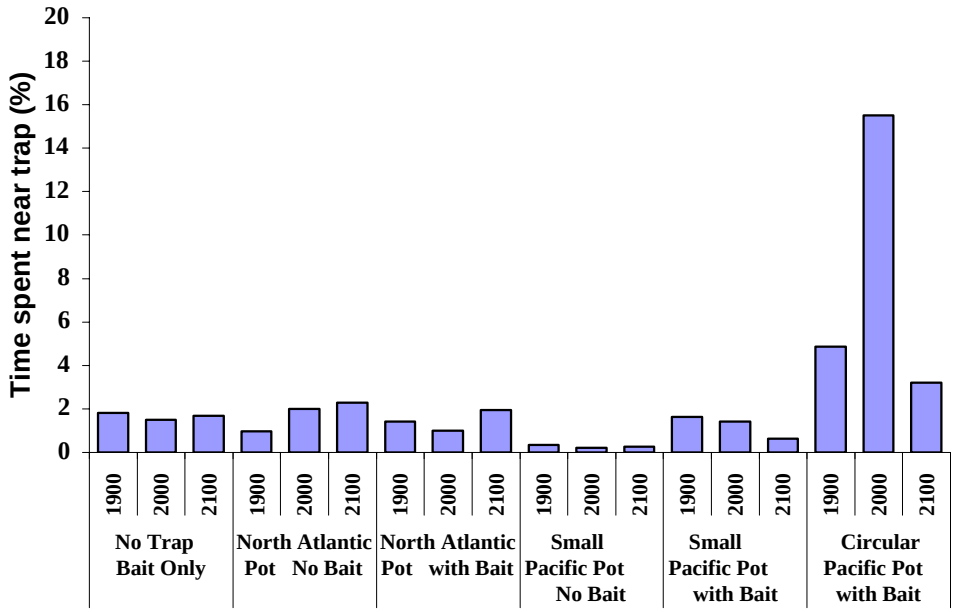


Figure 3.2. Histogram of time prawns spent in immediate vicinity of various baited and unbaited pot traps during 1 hour intervals of 3 hours video monitoring.

Table 3.1. Time prawns spent in immediate vicinity of the various pot traps at 1 hour internal during 3 hours of video recording. No prawns actually entered any of the pot traps.

Trap and Bait	Time	Prawns	Time Spent Near Trap (%)	Number of prawns trapped	
No Trap Bait Only	1900	7	1.81	0	Figure xx a
	2000	7	1.49		
	2100	7	1.68		
North Atlantic Pot No Bait	1900	7	0.98	0	Figure xx b
	2000	7	2.00		
	2100	7	2.28		
North Atlantic Pot with Bait	1900	7	1.42	0	Figure xx c
	2000	7	1.01		
	2100	7	1.96		
Small Pacific Pot No Bait	1900	7	0.35	0	Figure xx d
	2000	7	0.22		
	2100	7	0.27		
Small Pacific Pot with Bait	1900	7	1.62	0	Figure xx e
	2000	7	1.42		
	2100	7	0.63		
Circular Pacific Pot with Bait	1900	7	4.86	0	Figure xx f
	2000	7	15.51		
	2100	7	3.22		

Discussion

Although the patterns are similar for all treatments, prawns spend all their time swimming rapidly around the edges of the tanks following the walls. Of the pot trap designs tested only the circular North Pacific pot trap was of any attractiveness to the prawns. Overall though, as no prawns were trapped over the course of the experiment, none would appear to be suitable for trapping prawns.

There are a number of interpretations of these data. As there was limited directional flow information within the arena tank, as the pot traps took up a large proportion of the tank, there would be very little odor trail information. Optimized water flow past the chemo-attractant is essential for odor tracking (see introduction and discussion in Objective 1) and such conditions may not have been met. It is perhaps noteworthy that a purely chemo-attractant odor plume from a food source in one of the 4 holding tanks with water overflow feeding into one of four channels with distinctive flow into the main arena area of the tank did result in prawns being able to odor track the source (see Objective 1). There was little to no evidence to indicate odor tracking when either food in a mesh or a trap was positioned in the middle of the arena.

It is known that penaeid prawns will enter pot traps (see Racek 1955, 1959, King 1981, Barus 1989). It is not known, however, if the propensity for penaeid prawns to enter a baited pot trap is sufficient to make pot trapping an efficient harvest technology. Pot traps for Crustacea are designed taking into account the specific behavior of the animal in order to maximise the likelihood an animal would enter the trap. All of the pot traps trailed in this study were designed for cold-water pandalid shrimps. The behavior of these shrimps may vary significantly from that of tropical penaeid prawns (Barr and McBride 1967). Based on behavioural observation of penaeid prawns in captivity and from underwater video footage of their behavior about food bait in the wild, several pot traps designed could be developed that might increase the propensity for prawns to enter them. In addition, a design that minimizes the ability of prawn predators to enter a pot trap is essential. Previous studies on pot traps for penaeid prawns (see references above) have noted that whereas prawns may enter a trap they do not do so if other non-prawn animals have entered the pot.

BENEFITS AND ADOPTION

The beneficiaries have been:

The environmental agencies that initiated this investigation into the possibility of the development of an alternative harvesting technology for penaeid prawns and specifically a species-specific pot trap fishery.

The wild fishery sector, in the knowledge that it is unlikely in the medium term that a pheromone bait fishery can be develop as has been developed successfully for harvesting (as a form of biological control) terrestrial insects and is done in the blue crab (*Callinectes sapidus*) fishery sector in USA and the Caribbean rock lobster fishery.

The seafood sector, in that it is unlikely in the medium term that penaeid prawns will be caught by pot traps as is the case for prawns in the north Pacific and north Atlantic where such prawns fetch premium prices compared to trawled ones on niche international markets.

FUTURE DEVELOPMENT

This project was the first in a planned phase development towards a non-trawl harvest technology for penaeid prawns by the development of a species-specific pheromone pot trap fishery. In the first instance this project set out to demonstrate that it was possible to attract prawns to a point chemo-attractant source, especially that of a pheromone. In addition projects objectives were to extract, isolate and characterize penaeid prawn reproductive pheromones and to demonstrate that prawns will enter a pot baited with such a pheromone. Some progress has been made towards these objectives but much remains to be developed.

From a research perspective:

Further effort is required in the characterisation of the 23 female specific molecules preliminarily identified by mass spectrometry as being pheromone candidates.

In the absence of any bioassay directed pheromone chemical characterization employ a molecular biological approach in the isolation of chemoreceptors from prawns. There is now sufficient molecular sequence and structure data of chemoreceptors in global DNA databases to allow molecular strategies to be directed towards the isolation of penaeid prawn chemoreceptor structure from male antennules. Once this is known computer modelling of suitable molecules structures for the receptor can be investigated. This is one approach that would allow the identification of the molecular structure of the putative reproductive pheromone in the absence of a bioassay.

Demonstrate that a 'far-field' attractant pheromone is sufficient to attract a male penaeid prawn into a prawn in the absence of the further stimulus of a female contact pheromone. Alternatively to use the female 'near field' contact pheromone is conjunction with the far-field pheromone attractant.

From the fishing and seafood sector perspective:

Consultation with the seafood sector to identify whether any change, especially a decrease, in prawn harvest that may occur due to recent closures of significant regions of the Great Barrier Reef Marine Park should be met by wild catch, by aquaculture production or imports.

Establish whether an adult male only fishery is sustainable for the specific penaeid prawn species. Compare the impacts of such a targeted harvest with that of the adult male only mud crab established wild fishery.

From the environmental management sector perspective:

Consultation with the environmental organizations on whether an alternative harvesting technology of wild prawns or farm production is the most appropriate route to supply market demands for seafood that is considered to have human health benefits.

Evaluate whether the consequences of the development of a pot trap fishery for penaeid prawns would lead to equivalent environmental concerns as that of a trawl fishery. Pot trap fisheries for crustacean are well developed in the north Atlantic and also have their own environmental impacts.

PLANNED OUTCOMES

This project was the first stage towards an eventual goal leading to the development of alternative harvest technologies for penaeid prawns, especially in areas considered to be environmental sensitive to the putative impacts by prawn trawling on benthic ecosystems. Specifically the project examined the possibility of developing a species-specific pot-trap fishery by using pheromones as bait.

A second phase, as another application, and in direct association with regulatory agencies (Great Barrier Reef Marine Park Authority (GBRMPA) and prawn fishers (QCFO Trawl Committee), would undertake pilot trials of the use of the pheromone bait in pot traps in coastal waters. In the first instance these trials would utilise the pot designs used in the north Pacific and Atlantic prawn pot industry. These trials would examine catch rate in various habitats and topographies in association with GBRMPA and QCFO.

The pivotal outcomes for the first part of the development of a prawn pot trap fishery are:

a) The demonstration that prawns can be attracted to a point source of pheromone

Penaeid prawns can odor track chemo-attractants to a point source of food. The data generated from this project clearly demonstrates that there is ample evidence that a reproductive pheromone is of importance in the life history penaeid prawns. A significant sexual dimorphism exists with males having an acute sense of smell compared to females. This dimorphism only develops at the time of sexual maturity, indicating that the odor sensitivity, and possible discriminatory ability, of males is related to reproductive processes and presumable includes mate searching. Data was also obtained indicating that males have better developed brain centers for the processing of olfactory information. As penaeid prawns exhibit extensive searching during the hours of darkness it is very feasible that a male prawn would cross an odor plume of a female emitting a far-field reproductive pheromone to assist the male in locating the point source of the female. As successful mating is only possible within a very short time window, probably of only a few hours, a highly potent chemo-attractant in penaeid prawns is likely to be operational. Data was also obtained indicating that females contain many more sex specific putative pheromone-like compounds compared to males. However, due to the limitations of using a bioassay directed purification strategy in penaeid prawns by which to fully purify the pheromone its chemical identity remains unknown.

b) The demonstration that prawns can be enticed to enter a pot trap that has been baited with a pheromone

As a purified pheromone candidate compound was not produced, the demonstration that a penaeid prawn would enter a pot trap remains unanswered. However, from previous work and (see Background section) it is known that penaeid prawns, especially *P. japonicus,* will enter a food baited pot-trap. Penaeid prawns will readily go into a trap as long as there are no other organisms within the pot. As the semi-tropical and tropic areas are particularly rich in marine biodiversity, including predators, a pot trap design would be required that exploits specific aspects of penaeid prawn behaviour to increase the likelihood that they would preferentially enter the pot. Video recording of prawn behavior indicates that a trap that is slightly raised off the bottom is potentially suitable as prawns approach traps from the base and do not readily explore the height of the pot. An entrance to the periphery of the pot-trap base would need to be designed to physically exclude non-penaeid prawn shaped organisms. In addition, if there was added complexity to the internal compartments of the pot-trap, with the equivalent of a refuge, similar to the 'kitchen and parlour' design of pot-traps for the north Atlantic lobster fishery. As prawns will enter a food baited pot-trap is remains to be demonstrated that a pheromone bait is sufficient to do the same.

CONCLUSIONS

The project had three major objectives.

Objective 1: To quantify the attraction and specificity of pheromones from crustacea in experimental environments.

This objective had two main components. In the first instance, research was directed to the demonstration that penaeid prawns can detect pheromones, that they can produce pheromones and whether there is evidence for sex-specific detection and/or production of pheromones. It was found that male prawns are profoundly more chemo-sensitive than females. Male prawns have up approximately 150 chemoreceptor neurons per chemoreceptor sensilla making an estimated 231,200 chemoreceptors per antennules or over half a million chemoreceptors per male. Such a chemoreceptor array should be exquisitely sensitive to odors and pheromones. Males also have significantly larger areas of the brain, called the olfactory lobes, devoted to the processing of chemical stimuli compared to females. Attempts were made to develop an electrophysiological approach to assist in the identification of pheromone-like compounds from seawater samples. However, this approach was unsuccessful as no electrophysiological recordings could be made. Rosette glands, believed to be the source of far-field pheromones in Crustacea, were found in penaeid prawns.

For the second part of the object, research was devoted to the development of a suitable whole animal bioassay and the design of an 'olfactometer' arena aquarium in which to undertake experiments to detect odor tracking behavior. A flow-through system was developed in which a male prawn had the choice of 4 flow channels from which to choose. All water entering the aquarium was chemical and physically treated to remove organics and to produce a baseline seawater standard against which experimental pheromone containing seawater was tested. Each of the four channels either had normal seawater or female water, that included either females in a non-reproductive state or ones assumed to be releasing a pheromone. The basic behavior pattern of penaeid prawns in captivity is for profound activity during the hours of darkness with little or no activity during day light hours. A far-red video surveillance system was developed to track individual prawns in the aquarium arena. As penaeid prawns prefer to track along the edge of the aquarium, two of the four channels could not be used as test channels as they formed part of the tank walls and prawn tracking in these channels was 'normal' behavior. It was shown that penaeid prawns can odor track and locate a chemo-attractant source, such as food. However, it could not be demonstrated that male prawns can odor track putative

pheromone containing water. If a female is actually within the arena aquarium tank then males do exhibit distinct tracking to the female with extensive tactile contact. It was not certain whether male location of the female was far-field pheromone mediated, although there was extensive searching by males in sectors of the tank that they would not otherwise search. An 'olfactometer' system could not be developed for penaeid prawns that could be used as a bioassay for pheromones. It is highly desirable to have a bioassay directed pheromone strategy but this could not be developed for penaeid prawns as they do not possess readily distinguishable courtship or mating behavior as some other crustacea do, such as crabs and lobsters.

Objective 2: To develop methods suitable for isolating and concentrating pheromones from crustacea, especially penaeid prawns.

The isolation of a highly dilute pheromone is not readily achievable from large volumes of seawater. Part of the problem is that the concentration of the pheromone is unknown so it is impossible to actually predict what order of magnitude of purification is required. Assuming dilutions of up to 10^{-18}M the technical challenges of purifying such a dilute chemical from seawater are significant. We could not develop a satisfactory method that could isolate a putative pheromone from large volumes of seawater in a flow-through system. In addition, a method to isolate the putative pheromone from batch processing of static aquarium water holding female prawns in a physiological state that was expected to contain pheromones did not produce any evidence of chemical candidates. We modified and further developed a technique that allowed the direct collection of urine from penaeid prawns. As it is assumed that far-field pheromones are released in the urine it is the most concentrated source of pheromone. Analysis of urine by mass spectrometry revealed that whereas there were only 15 male specific chemicals there were 133 female specific chemicals. Of these only 1 compound was found in more that one male whereas the evidence indicates that over 23 unique chemicals exist in female urine. These compounds are possible reproductive pheromone candidates. Within the life of this project, the full chemical characterization of these candidates was not successful. Alternative identification methods for pheromone characterization in the absence of a bioassay directed method, include a molecular biological approach in which the actual sequence of chemoreceptors of prawns can be determined. If the chemoreceptor structure is known then the pheromone molecular structure that can bind to them can be deduced.

Taking into account the profound sexual dimorphism in odor tracking sensitivity in males compare to females and the presence of unique molecules in adult females, there is strong evidence to indicate that penaeid prawns do use far-field pheromone communication. It is not known if the pheromone tracking ability of prawns is sufficient to entice them into a pheromone baited pot-trap.

Objective 3: To identify a mechanism for manufacturing a bait incorporating these novel attractants.

As a penaeid prawn reproductive pheromone was not characterized, and hence could not be supplied as a pure compound for inclusion into a bait, this objective did not progress sufficiently. However, a number of slow release polymer compounds exist which would allow the gradual release of an embedded chemical over time (Nussinovitch 2003). If a pheromone could be produced in sufficient quantities it is likely that it would be feasible to have an artificial pheromone bait that would function over time in the marine environment.

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APPENDICES

Appendix 1: Intellectual Property

No patentable inventions or processes have been developed as part of this project. General biological information may be considered public domain as it has benefit in contributing to any future debate on the development of alternative harvest technologies, especially pot-trap fisheries, for crustacea in general and specifically for penaeid prawns.

Appendix 2: Staff engaged in the Project

Name	Role	Institution
Dr Mike Hall	PI	AIMS
Mr Neil Young	Experimental Scientist	AIMS
Professor David Sandeman	Neurobiology	UNSW
Dr Renate Sandeman	Histology	UNSW
Dr David Sheumack	Chemist	Hanitro Pty
Mr Matt Kenway	Manager, Aquaculture Facility	AIMS
Mr Don Booth	Experimental Scientist	AIMS
Mr Matthew Salmon	Experimental Scientist	AIMS
Mr Rick Willis	Mass spectrometry Manager	AIMS
Dr Cederic Robillot	Mass spectrometry	AIMS
Ms Liz Howlett	Report edit and production	AIMS
Mr Tim Simmonds	Report production	AIMS
Dr Kate Wilson	Aquaculture Team Leader	AIMS

Appendix 3: Project Outputs

Journals

Young, N.D., Sandeman, D. and Hall, M. (submitted) Sexual dimorphism in the development of antennule aesthetascs of *Penaeus monodon* (Decapoda: Penaeoidea). J. Crust. Biol.

Media releases

Television:

14 April 2002, ABC TV Darwin, Brisbane, Sydney, 7 pm news bulletin New study looks at fishing for prawns using pheromones. Interview with/footage of Mike Hall.

Radio:

April 10 – 11 2002, ABC Radio (North Qld) *Northern Extra* , ABC Radio (Wide Bay), 6.30 am, 8.30 am news, ABC Radio (West Qld), 8.30 am news, ABC Radio (Far North, Cairns), 6.30am, 7.30am, 8.30 am news, ABC Radio (North Qld), 8.30 am, ABC Radio (Tropical North, Mackay), 8.30am AIMS scientists use sex to lure prawns into traps. Mike Hall interviewed.

Conference Proceedings

Oral Presentations:

AMSA Townsville 2001 July 3-6 "Changes in the Marine Environment"
Title: Chemo-attractants as a possible alternative harvest method for penaeid prawns.

AMSA Fremantle 2002 July 7-9 "Marine Biodiversity"
Title: Chemo-attractants as a possible alternative harvest method for penaeid prawns.

Poster:

AMSA Brisbane 2003 July 9-11 "Marine Biocomplexity"
Title: Characterising chemo-attractants from penaeid prawns