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The behavioural function of pheromones in crayfish

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by

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Abstract

Pacifastacus leniusculus and *Procambarus clarkii* are highly invasive freshwater crayfish and are having detrimental impacts on native species and habitats throughout Europe. The application of pheromone baits have been proposed as a way of increasing trap efficiency for population control, however the chemical identity of crayfish pheromones is unknown. An incomplete understanding of chemical communication has delayed progress in the development of appropriate bioassays. This thesis therefore focused on researching the natural context of chemical signalling by crayfish, including signal delivery and receiver response.

Urine release by male and female crayfish was found to coincide with aggressive behaviours rather than reproductive behaviours. Female urine release was essential for initiating mating, with males detecting female receptivity by spying on hormones and metabolites released with threat signals. Physiological indicators of reception included a brief cardiac and ventilatory arrest followed by an increase in rate. Both behavioural and physiological responses formed the basis of a novel assay design.

During courtship male crayfish do not appear to advertise by urine signals. This raised the question of whether chemical signals were important for female assessment of the quality of size-matched males. When given a free choice, females could not distinguish dominant and subordinate males through chemical signals alone. This suggests that females either use other criteria (e.g. size) for mate choice or perform cryptic post-copulatory mate choice.

Blocking natural urine release of crayfish, which had previously fought to establish dominance, and artificially introducing urinary signals proved an effective bioassay for investigating the mechanisms of dominance hierarchy formation. Urine from the dominant male was the key factor in establishing dominance relationships. In the absence of dominant urine, subordinate males were less likely to retreat from aggressive bouts and fights were more intense.

The mechanisms of signal delivery during agonistic encounters were investigated by measuring ventilatory activity. Increased ventilation rate was associated with highly aggressive behaviours and urinary signalling. This indicated crayfish create gill currents to disperse signals and increase transfer efficiency from sender to receiver.

This thesis sheds light into the mechanism of chemical communication in crayfish and provides the basis for future bioassay guided purification of crayfish pheromones.

Chapter 1

Introduction

1.1 Biological communication

The exchange of signals within ecosystems can have a large impact on the population dynamics and ecology of organisms, through regulation of essential survival processes such as mate attraction, resource competition, foraging strategies, navigation and predator avoidance (Harper 1991; Krebs & Davies 1993; Bradbury & Vehrencamp 1998). Despite this, one of the most contentious issues in animal behaviour is how to define communication, with multiple definitions present in the literature (Hauser 1997; Bradbury & Vehrencamp 1998; Maynard Smith & Harper 2003; Scott-Phillips 2008). Modern definitions of communication emphasise that both signal and response need to be adaptive (Maynard Smith & Harper 2003; Scott-Phillips 2008). The adaptationist approach by Scott-Phillips defines a signal as any act or structure that (i) affects the behaviour of other organisms (ii) which evolved because of those effects, and (iii) which is effective because the receiver's response has evolved to be affected by the act or structure (Maynard Smith & Harper 2003; Scott-Phillips 2008). The requirement that a signal has evolved because of its effect on others, distinguishes a 'signal' from a 'cue' (Maynard Smith & Harper 2003). Cues can be defined as any feature of the world, which is animate of inanimate, that can be used by an animal as a guide to future action (Hasson 1994; Maynard Smith & Harper 2003). Concise definitions can be important as they contribute to the understanding of systems and help to focus future discussions.

Signals can be conveyed using visual, chemical or mechanical stimuli, with communication between individuals often relying on multiple signalling pathways working synergistically (Bruski & Dunham 1987; Hughes 1996; Rowe 1999; Acquistapace et al. 2002; Diaz & Thiel 2004; Hebets & Papaj 2005).

1.1.2 Chemical communication

Chemical signalling is one of the oldest forms of communication, and is utilised by animals ranging from simple bacteria to large vertebrates (Johansson & Jones 2007; El-Sayed 2008). Early life forms utilised chemical cues to detect food, which is still the primary function of most chemoreception organs (Bradbury & Vehrencamp 1998). Once individuals' could distinguish chemical cues of food from those released by conspecifics (from metabolic waste) a primitive communication system could evolve. Stacey and Sorenson (2005) have proposed that the evolution of chemical communication involves three distinct stages (i) ancestral – individuals release essential metabolic products which do not influence receivers (ii) spying – selection causes

receivers (conspecifics) to detect and respond adaptively to the released chemicals, which can now be considered a pheromonal cue (iii) communication – the releaser benefits from cue production and develop specialisations to produce it more effectively, this adaptation results in signal (rather than cue) production (Stacey & Sorensen 2005). By gaining benefits from the cue the receiver will increase its sensitivity to the pheromone (Bossert & Wilson 1963). The evolution of specialised chemoreceptors allows receivers to detect chemicals, even at extremely low concentrations (Laverack 1963; Grasso & Basil 2002). For example, decapod crustaceans can have millions of chemosensory neurons and specialised receptors which enable detection of chemical signals (Derby & Steullet 2001; Belanger & Moore 2006).

Chemical cues and signals are considered vital for co-ordinating important processes such as foraging (Shelton & Mackie 1971), predator avoidance (Petranka et al. 1987), migration (Lohmann et al 1999), brood care (Little 1976), larval settlement cues (Clare & Matsumura 2000) and regulating social interactions – including agonistic interactions, mating and sexual selection (Moore et al 1995; Rollmann et al. 2000; Atema & Steinbach 2007). Despite the importance of chemical communication, most studies have focused on direct interactions between animals, which rely on visual stimuli and have not considered the potential impacts of far-field chemical cues (Brönmark & Hansson 2000).

There are various terms and concepts which distinguish forms of chemical signals based on their behavioural function and effects on receiving animals. Chemicals which mediate interactions between organisms are called semiochemicals. Their effect may be intra-specific, pheromones, or inter-specific, allelochemicals. Pheromones were first defined by Karlson and Lüscher (1959) as cues which elicit a specific response in the receiver, either a definite behavioural or developmental process. Advances in chemical ecology studies have indicated this definition needs broadening to include a wide range of behavioural responses and chemical cues (Wyatt 2002; Stacey & Sorensen 2005). Pheromones can be eavesdropped between species, resulting in potential costs (kariomone cues) or benefits (allomone cues) to the signaller in relation to the receiver (Dicke & Sabelis 1988). Mutualisms may form between different species resulting in chemical signals (synomones) which are beneficial to both the emitter and receiver (Dicke & Sabelis 1988; Agosta 1992; Wyatt 2002). The function of pheromones is

determined by life history strategies, population dynamics and specific behaviours of individual species.

1.1.3 Aquatic chemical signalling

Although vision can play an important role in communication for terrestrial animals, visual stimuli can be constrained in aquatic environments by poor light transmission, especially in turbid waters. Chemical communication has therefore been found to play an integral role in regulating the social interactions of many aquatic organisms (Rittschof 1993; Brönmark & Hansson 2000; Atema & Steinbach 2007). For example, pheromones play a key role in co-ordinating the synchronous release of gametes by male and female ragworms, *Nereis succinea* (Hardege 1999). Synchronisation of spawning is particularly important as reproduction is the terminal event in the ragworm lifecycle.

In the aquatic environment animals have been described as "leaky bags", where chemicals are emitted as a result of necessary metabolism where odour sources include the gills, urine or faeces (Atema 1985; Atema 1995; Stacey & Sorensen 2005). The chemical composition of cues released from metabolic processes can change depending on the internal hormonal state of the signaller, which can be exploited by conspecifics or predators (Stacey & Sorensen 2005). Multiple stimulatory chemicals are therefore naturally present in aquatic environments and create complex temporal and spatial dynamic patterns, which can be highly influenced by ambient currents (Atema 1996).

Animals need to maximise their ability to receive chemical signals/cues to make informed ecological decisions about suitable foraging and mating strategies, predator avoidance and habitat selection. Individuals therefore must distinguish odour types and their sources from background noise (Atema 1985; Zimmer-Faust et al. 1995; Wolf et al. 2004). This can be achieved through recognition of a signal's unique properties or high concentration. Individuals may locate an odour source through guidance using directional movement such as mean flow (rheotaxis) or odour guided movements (chemotaxis), which can be influenced by changes in plume dynamics and chemosensory ability (Zimmer-Faust et al. 1995; Grasso & Basil 2002; Wolf et al. 2004). The ability of a receiver to detect a chemical stimulus can be influenced by environmental conditions, such as substrate and hydrodynamics, as well as the quality of the signal itself (Moore & Grills 1999; Burks & Lodge 2002; Kozlowski et al. 2003;

Hazlett et al. 2006). Chemical stimuli can become more homogenous from the point of release which is problematic for far field attraction (Atema 1985).

The detection of chemical signals by receivers can be enhanced by signallers altering their release strategy to increase the potential for the receiver to detect the pheromone (Harper 1991). This can be especially important due to the slow diffusion of chemicals in an aquatic environment. Some decapod crustaceans are capable of manipulating their external environment by creating currents which can transport molecules towards opponent chemoreceptors for assessment (Atema & Voigt 1995; Breithaupt 2001). For example, lobsters, *Homarus americanus*, are capable of using scaphognathites within the gill chambers to create currents which can reach 7 body lengths (Atema 1985). In addition to gill currents, lobsters can fan the exopodites of the maxillipeds (the fan organs) to draw water towards the antennules for chemical sampling (McPhie & Atema 1984).

1.1.4 Aquatic chemical signalling – crustaceans

Crustaceans are one of the most widely studied group of aquatic animals; however there has been limited success in identifying pheromones used in communication, despite chemical signals being involved in the regulation of social activities (Atema & Steinbach 2007).

Crustaceans readily fight over resources and pheromonal signalling has been found to play a key role in mediating dominance fights (Atema & Steinbach 2007; Breithaupt & Eger 2002; Karavanich & Atema 1998a). Lobsters, *H. americanus*, are capable of individual recognition, with losers retreating and showing low levels of aggression during repeated fights with familiar opponents (Karavanich & Atema 1998a). Memory of these encounters can last between one and two weeks. Prevention of urine release or chemoreception demonstrates that individual recognition is mediated by chemical signals (Karavanich & Atema 1998b). The hermit crab, *Pagurus longicarpus* can chemically recognise individual conspecifics. When exposed to odours from familiar opponents individuals can discriminate large crabs in high quality shells from smaller crabs in low quality shells (Gherardi et al. 2005). The association between the odours of individual crabs with the quality of its shell can quickly change if shell quality changes (Gherardi et al. 2005).

As well as being important regulators of agonistic behaviours, pheromones can play a key role in mediating reproductive behaviours. The function of crustacean sex pheromones may be dependant on the life history strategy of the species studied. Some crustaceans, including the crab *Carcinus maenas*, and the lobster *H. americanus*, are soft shell maters. This is where mating only occurs following a female moult. Prior to moulting the male will often guard the female. It is intuitive that these animals use a sex pheromone as mating is restricted to a short time period and moulting is a very vulnerable time for the female. In lobsters, intermoult females can be rejected by males, possibly as a result of not possessing the pheromone indicative of the correct female moult stage (Atema & Engstrom 1971).

Crayfish are hard shell maters and as such breeding is not restricted to the female moult cycle. Crayfish can reach high population densities and it is therefore unlikely that distance pheromones commonly play a key role in co-ordinating mating activity. However some crayfish species have a short breeding season (e.g. *Pacifastacus leniusculus*) and consequently sex pheromones which trigger and control specific courtship behaviours may be present. Recent studies of *P. leniusculus* have shown that males are stimulated by odours from receptive female crayfish (Stebbing et al. 2003a) but not odour of juveniles.

There are multiple studies which provide evidence for the existence of sex pheromones in crustaceans. The presence of a female sex pheromone was first demonstrated in the crab *Portunus sanguinolentus* (Ryan 1966). Subsequent studies have shown multiple crab species utilise sex pheromones, such as the blue crab, *Callinectes sapidus* (Gleeson 1982), the helmet crab, *Telmessus cheiragonus* (Kamio 2000) and the Chinese mitten crab, *Eriocheir sinensis* (Herborg et al. 2006). The use of sex pheromones has also been demonstrated in lobsters, *Homarus americanus* (Atema & Engstrom 1971), rock shrimp, *Rhynchocinectes typus* (Diaz & Thiel 2004) and crayfish, *P. leniusculus* (Stebbing et al. 2003a).

Although multiple crustacean species have been shown to use sex pheromones to co-ordinate mating behaviours, to date only two pheromones have been chemically identified. Female hair crabs, *Erimacrus isenbeckii*, use a mixture of ceramides (lipid secondary messengers used in intracellular signalling system), to elicit mating behaviours (Asai et al. 2000), whilst the shore crab's, *Carcinus maenas*, sex pheromone

is uridine-di-phosphate (Bublitz 2007; Fletcher 2007), which is linked to chitin biosynthesis.

In order to discover the identity of more aquatic pheromones multidisciplinary studies utilising complimentary biological and chemistry techniques need to be employed.

1.2 The chemical nature of pheromones

1.2.1 Pheromone identification

Despite over 40 years of research into aquatic pheromones there are currently very few identified water-borne pheromones. This has constrained our knowledge on aquatic chemical communication, with researchers having to draw restricted conclusions as to the behavioural significance of their work.

The first mate attractant molecule to be identified was that of the silkworm moth (Butenandt et al. 1959) and since its discovery a tremendous research effort has gone into unravelling the molecular structures of insect chemical signals, mainly due to their application in integrated pest management. In contrast, there has been limited success in identifying the chemical structure of water borne cues/signals, which are harder to detect and measure than their visual and acoustic counterparts. Identified aquatic sex pheromones include those of the ragworm, *Nereis succinea* (Hardege et al. 2004), the shore crab, *C. maenas* (Bublitz 2007) and a number of fish species including the goldfish, *Carassius auratus* (Stacey & Sorensen 2005).

The chemical nature of pheromones can be related to their signalling function with solubility being the key factor for aquatic pheromones. The method used to identify pheromones is dependant on the chemical properties of the compound investigated. However, independent of the species studied there are similarities in the identification stages involved (Hardege et al. 2002; Wyatt 2002; Derby & Sorensen 2008). Identification of pheromone cues relies upon separation of active chemical components from complex mixtures. Separation can be based on a number of characteristics including molecular mass, molecular charge and solubility in solvents of different polarity. The resultant fractions can then be tested for bioactivity using either a behavioural or physiological assay. There are several problems that can occur at each stage of identification, including the degradation of chemical components or loss of material during separation and purification.

1.2.2 Bioassay development

Bioassay guided fractionation is an important technique for identifying the bioactive molecules of aquatic pheromones as it makes no assumptions about their chemical nature, this is especially important where there is no candidate chemical to focus analytical studies. Therefore the first stage in the identification of aquatic pheromones involves developing a reliable assay which can be used to assess an individual's receptivity to different fractions of the original complex mixture substance. Assays should be biological relevant, methodologically simple and ideally evoke a specific response which reflects the receptivity of animals to natural chemical stimulation. For example a bioassay used in sex pheromone identification should be based on a specific sexual response in the subject animal following chemical stimulation (such as cradle carrying in crabs, see Kamio et al. 2000; Hardege et al. 2002). Behavioural measures of receptivity need to be reproducible in order to form the basis of a suitable assay.

Physiological assays can also provide a quick and reliable method to identify chemical stimulation. For example, electroantennograms (EAGs) and electroolfactograms (EOGs) are important tools used for identification of insect and fish pheromones, respectively. These assays use recordings of the electrical impulses from the chemosensory cells as they are stimulated by pheromones. Electrophysiological techniques only indicate sensitivity to a compound, rather than an individual's behavioural response. Alternative physiological assays can be based on standardised physiological responses, such as changes in heart rate and/or ventilation rate to chemical stimulation (Li et al. 2002; Murphy & Stacey 2002).

In order to design an efficient and reliable assay it is important to observe and identify the behavioural function of the pheromone in natural situations. Visualisation of signal release using dye can help determine the natural timing of signal delivery to the receiver (Breithaupt & Eger 2002) and focus assay design.

Once a suitable assay has been designed then sufficient amounts of the chemical signal needs to be collected, this needs to compensate for the inevitable loss of material during subsequent analysis.

1.2.3 Chemical analysis

The major challenge following sample collection is to identify the active compound(s) within a complex mixture. At this stage if a candidate substance is proposed as the pheromonal cue analytical chemistry techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC) and mass spectroscopy (MS), can be employed as identification techniques. Most studies, however, involve the identification of novel pheromones. Therefore chemical mixtures must be purified by fractionating/separating components from the natural mixture they occur, on the basis of their chemical and physical attributes. Each fraction is then tested, using a suitable assay, for biological activity and fractionated further until the pheromone can be identified using analytical techniques (e.g. HPLC, GC, and MS).

The ability to link the fractionation of compounds with real time bioassays (EAGs) has led to the identification of numerous pheromones used by insect species (El-Sayed 2008). This technique is not employed for aquatic organisms and unlike terrestrial organisms; few aquatic pheromones have been identified (Zeeck et al. 1988; Hardege et al. 1996; Clare & Matsumura 2000; Sorensen & Stacey 2004).

1.3 The use of pheromones to control invasive species

The use of pheromone baits to lure and trap individuals have been extremely successful in trapping insect pests in terrestrial systems (Agosta 1992) but little is known about their application in aquatic environments (Corkum 2004). An exception is the use of pheromone traps in the integrated management scheme for invasive sea lamprey (*Petromyson marinus*) in the Great Lakes of North America. Sea lamprey were introduced into the Great Lakes via the Wellard Canal in the 1920's and are considered pests due to their parasitization of economically important fish stocks (Smith & Tibbles 1980). Trials using pheromone traps are in their initial stages following recent identification of the active chemical components of the sea lamprey migratory pheromone and the sex pheromone (Li et al. 2003; Sorensen & Vrieze 2003).

Transportation of crustaceans out of their natural ranges has resulted in many species acting as pernicious invaders (Cohen et al. 1995). For example crayfish are highly adaptable and have become established pests in a variety of freshwater environments, causing the decline of native species and destroying indigenous habitats (Lodge et al. 1994; Guan & Wiles 1997; Lodge 2000). As a result there is heightened interest in

identifying the chemicals utilised by crayfish to regulate their social activities. If crayfish sex pheromones could be identified it is hoped that methods can be developed to control numbers using pheromone trapping techniques (Stebbing et al. 2004). Recent field trials have indicated female odours may provide an effective bait during the breeding season, although its effectiveness in comparison to food baits is still questioned (Stebbing 2005; Stebbing et al. 2004).

Identification of crayfish sex pheromones could improve efficacy of pheromonal baits, however attempts to identify sex pheromones and agonistic pheromones of invasive crayfish have been hindered by the lack of a suitable bioassay. Previous bioassay designs have focused on the use of non-sexual behaviours (activity, handling airstone) by male crayfish (*P. leniusculus*) to water conditioned by mature, receptive, females (Stebbing et al. 2003a; Belanger & Moore 2006). Alternative bioassay methodology therefore needs to be developed, potentially using both behavioural and physiological measurements, to assess sex-specific responses to pheromonal stimulus.

Before a suitable bioassay and analytical chemistry techniques can be employed for the identification of invasive crayfish pheromones it is imperative to fully understand the behaviour, life history characteristics and social activities of the crayfish species studied.

1.4 Biology and ecology of crayfish (The Astacoidea)

Crayfish are the largest, mobile freshwater invertebrate and as such can have a discernable biological impact on the environment (Creed 1994; Holdich 2002). There are 540 recognised species of crayfish, classified into two superfamilies; Astacoidea and Parastacoidea. Astacoidea is split into two families the Astacidae and Cambaridae, both of which contain species which are now considered pests away from their natural ranges, in particular signal crayfish, *P. leniusculus* (astacid), and red swamp crayfish, *Procambarus clarkii* (cambarid).

Crayfish inhabit a wide range of habitats, from lentic and lotic waters of varying salinity to semi-terrestrial environments, with species-specific preferences for certain conditions (Nystrom 2002; Welch & Eversole 2006). Red swamp crayfish are secondary burrowers which are ideally adapted to temporary lentic systems that periodically flood. Signal crayfish depend less on burrow systems and have a preference for slow flowing stream

habitats (Hogger 1988). However, both signal crayfish and red swamp crayfish are highly adaptable and can therefore inhabit heavily modified habitats.

Crayfish are considered a keystone species due to their opportunistic and omnivorous feeding habits (Creed 1994). Feeding is influenced by season, temperature and body size (Guan & Wiles 1998; Renz & Breithaupt 2000). Crayfish feed on a range of foods, including species of submerged macrophytes, fish, filamentous algae, macro-invertebrates, fish eggs and detritus (Nystrom, 2002). Like many other crustaceans, crayfish can seek out food with the highest energy content using chemical cues (Zimmer-Faust 1987; Corotto & O'Brien 2002; Nystrom 2002). Crayfish are cannibalistic, which is especially common in the summer and winter, when animals are injured or freshly moulted (Guan & Wiles 1998).

1.4.1 Morphology and growth

Male crayfish moult more frequently than females with growth resulting in sexual dimorphism. In comparison to females, male crayfish have larger chelae in relation to their carapace length, whilst females possess a broader abdomen to accommodate eggs (Reynolds et al. 1992). The sexes can also be distinguished by the presence of male copulatory stylets on the ventral surface which are used to deposit spermatophores on the female (figure 1.1; Holdich 2002). Growth rate and size varies between different habitats and between species. For example *P. leniusculus* can grow to total lengths of 84-140mm and are slightly larger than *P. clarkii* which reach lengths of 84-135mm (Lewis 2002).

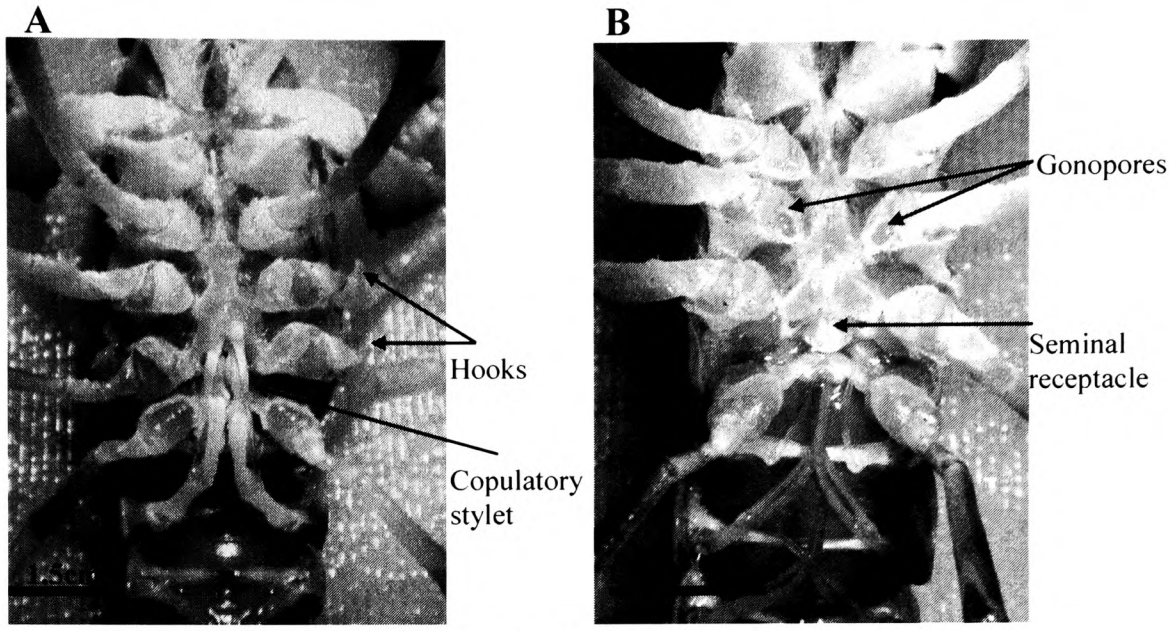

Figure 1.1: Ventral views of A) Form I male and B) female *P. clarkii*. Copulatory stylets are evident for male crayfish, with hooks on the second and third walking legs. The stylets are used to deposit spermatophores on the cornified seminal receptacle, the *annulus ventralis* located on the female above the 4th walking leg.

Cambarid crayfish, such as *P. clarkii*, undergo cyclical ecdyses between reproductive and non-reproductive forms depending on their maturity and mating history (Huner 2002). Sexually active males are described as Form I individuals and have large chelae, cornified gonopodia and prominent copulatory hooks on the base of the 3rd and 4th walking legs, which are used to secure the female during mating (figure 1.1). Following mating success males moult back to the inactive Form II morph, displayed by immature males. It is uncertain if females return to non sexual forms following brooding. Unlike cambarids, astacid crayfish, such as *P. leniusculus*, undergo an irreversible change to maturity aged between one – three years and do not possess copulatory hooks.

1.4.2 Life history strategies

The type of life history strategy employed by animals is influenced by the energy budget and resources available in the surrounding environment. For crayfish this results in life history strategies being heavily influenced by the water temperature of their native environments. Red swamp crayfish inhabit warm, sub tropical regions and display 'r' selected characteristics like rapid maturation, high fecundity and short life span. Signal crayfish, however, are constrained by low temperatures and consequently displays 'k' selected characteristics such as longer life expectancy, slow seasonal

growth and iteroparous production of relatively low egg counts (table 1.1; Reynolds 2002).

Table 1.1: Summary of life history characteristics for astacid (*P. leniusculus*, *A. pallipes*) and cambarid (*P.* clarkii) crayfish found in Europe (Reynolds et al., 1992; Reynolds 2002). *P. clarkii* shows typical r selected characteristics whereas astacid species are constrained by temperature in their natural habitats and display k selected parameters.

	Procambarus clarkii	***Pacifastacus leniusculus***	***Austropotamobius pallipes***
Carapace length (mm)	70	75	60
Carapace length at maturity (mm)	45	30	22-25
Life span (months)	12 – 18	48 – 100	72 – 168
Egg diameter (mm)	2	2.5 – 3	2.3 – 3.3
Pleopodal egg no.	400 – 600	110 – 250	20 – 65
Incubation period (days)	20	240	260

Red swamp crayfish have a protracted breeding season and are believed to mate whenever they locate a sexual partner (Ameyaw-Akumfi & Hazlett 1975). In contrast, *P. leniusculus* has a short breeding season of two/three months, with maximal reproductive effort in October (Lewis 2002). In astacid crayfish, mating is reliant on a fall in temperature and decline in photoperiod. This could result in pheromone production along with maturation of the ovaries (MacRae & Mitchell 1995). Timing of spawning is controlled by hormones that are regulated by photoperiod and temperature (Dube & Portelance 1992).

1.4.3 Crayfish in the UK and Europe

There are 5 native astacid species of crayfish in Europe (table 1.2), three from the *Astacus* genus (*Astacus pachypus*, *Astacus astacus*, *Astacus leptodactylus*) and two from the *Austropotamobius* (*Austropotamobius pallipes*, *Austropotamobius torrentium*). Members of the *Austropotamobius* genus are located from Spain to the Balkans and on the British Isles while members of the *Astacus* occur from France to the Ural Mountains, including southern Scandinavia (Taylor 2002; Souty-Grosset et al. 2006). *A. pallipes* (white-clawed crayfish) is the only species native to the British Isles.

1.4.3.1 *White-clawed crayfish in the UK*

There is only one species of native crayfish in the UK, the white-clawed crayfish (*A. pallipes*). White-clawed crayfish are patchily distributed, both between water bodies and within the local habitat. The cause of distribution patterns can be a combination of natural restrictions (habitat quality) and additional threats and pressures (introduction of non-indigenous crayfish, disease, pollution) (Holdich 2003).

Native species have come under threat from anthropogenic sources of disturbance such as habitat modification, pollution and introduction of non-indigenous crayfish species (Souty-Grosset et al. 2006). Declines in white-clawed crayfish population numbers (figure 1.2) has led to its protection under Annex II of the EU Directive on the Conservation of Natural Habitats and of Wild Fauna and Flora (92/43/EEC). In addition the UK has its own Biodiversity Action Plan/Species Action Plan for white-clawed crayfish where protected species need to be taken into account by planning authorities and efforts are being made to increased the species range (number of occupied 10km grid squares) by 2030. To help achieve these aims, efforts are being made to translocate, introduce and re-introduce populations of white-clawed crayfish populations whilst developing methods to control/eradicate threatening signal crayfish populations (Peay & Hiley 2001; Peay 2002; Peay 2003; Peay 2004).

Figure 1.2: White-clawed crayfish (*A. pallipes*) distribution in 10 km squares in the UK, complied from available datasets on the National Biodiversity Network (www.searchnbn.net). (A) 1979 – 88 (B) 1999– 2008. The maps show the decline in *A. pallipes* since the introduction and spread of invasive crayfish species in the 1970's.

1.4.3.2 *Non-indigenous crayfish*

Anthropogenic introductions from aquaculture, the aquarium industry and recreational activities have distorted global crayfish distributions (Lodge 1993). Non-indigenous crayfish have been introduced in multiple European countries, deliberately, as food sources for the local restaurant trade (table 1.2; Henttonen & Huner 1999). Introduced stocks can have positive impacts such as an economic benefit for crayfish farmers, diversification of agriculture to include astaciculture, restocking of lakes with disease resistant species in Finland and Sweden, aiding academic research into crayfish behaviours and helping to manage lakes which are overgrown by water plants (Henttonen & Huner 1999). However, despite these positive impacts in the majority of cases alien crayfish introductions are having highly detrimental impacts, which can outweigh the benefits.

Table 1.2: List of the eight non-indigenous crayfish species which have been introduced and have become established in Europe (Souty-Grosset et al. 2006). The number of invasive species present has doubled within the last five years (Taylor 2002; Souty-Grosset et al. 2006). There is a potential for further species to be introduced accidentally through the aquarium trade.

Indigenous Species	**Non Indigenous Species**
Astacus astacus	*Pacifastacus leniusculus*
Astacus leptodactylus	*Cherax destructor*
Astacus pachypus	*Orconectes immunis*
Austropotamobius torrentium	*Orconectes limosus*
Austropotamobius pallipes	*Orconetes virilis*
	Orconectes rusticus
	Procambarus clarkii
	Procambarus sp. Marmorkrebs

Introduced populations have been blamed for causing the degradation of freshwater habitats through negative impacts which include; displacement of native crayfish populations and amphibians, physical damage to the banks of water bodies through burrowing activity, reduction of fish stocks and transfer of disease (Holdich 1999; Holdich 2002). With complex trophic roles, crayfish have the capacity to control the abundance of macrophytes and invertebrates in lentic and lotic environments (Hogger 1988; Creed 1994; Lodge et al. 1994) and the decline of multiple freshwater species has

been attributed to crayfish invasions (Nyström & Strand 1996; Guan & Wiles 1997; Nystrom 2002; Dorn & Wojdak 2004; Bramard et al. 2006).

Non indigenous crayfish are now considered the major causes of native crayfish species decline with up to ½ of the world's crayfish species being currently at risk (Taylor 2002). Exotic species have the capacity to competitively exclude many indigenous crayfish populations; either by reproductive interference (Westman et al. 2002), out-competing for limiting resources (Soderback 1994; Usio et al. 2001) or by acting as vectors for the crayfish plague, *Aphanomyces astaci*, to which they are immune but can kill native species within two weeks of contraction (Vorburger & Ribi 1999; Souty-Grosset et al. 2006). Species replacement between alien and endemic crayfish can result in changes to ecological processes due to how different species process riparian food sources (Usio et al. 2006). Invasive species appear to be more opportunistic feeders than native species, readily switching to novel prey (Renai & Gherardi 2004) giving an advantage in a changing environment.

Five invasive species are now located on the British Isles with *P. leniusculus* and *A. leptodactylus* forming harvestable populations (Holdich & Rogers 1997). *A. astacus, P. clarkii* and *O. limosus* have restricted ranges in southern England (Souty-Grosset et al. 2006). Signal crayfish are the most widespread invader in the U.K. (figure 1.3). The potential range expansion of established river populations can reach 2.4 $kmyr^{-1}$, with maximum movements in mid-summer at 13-120 mdy^{-1} (Light 2003; Bubb et al. 2004; Bubb et al. 2005).

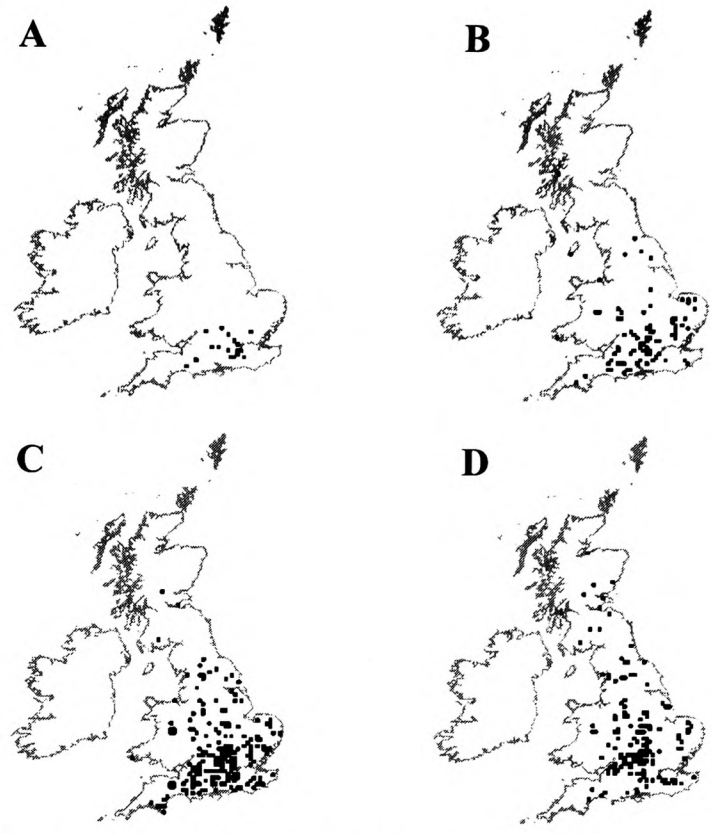

Figure 1.3: Signal crayfish (*P. leniusculus*) distribution in 10 km squares in the UK, complied from available datasets on the National Biodiversity Network (www.searchnbn.net). UK (A) 1969 – 78, (B) 1979 – 88 (C) 1989 – 1998 (D) 1999–2008. The maps show the spread of invasive crayfish populations since their introduction for the restaurant trade in the 1970's.

Non-indigenous crayfish species could potentially fill the role of keystone species left vacant by native species. However, alien crayfish can exert diverse, and often highly negative, impacts on their environment in comparison to their native counterparts. Native crayfish can also be regarded as having economic and cultural significance to a range of stakeholders and public groups (Holdich 2002). Eradication of invasive species in an attempt to restore native communities can help prevent the ongoing homogenisation of global biota (Lodge 1993).

1.4.4 Controlling invasive crayfish species

Management strategies to control invasive crayfish populations need to be species-specific and take into account any underlying genetic considerations to prevent further decline of vulnerable populations (Grandjean et al. 1997; Gouin et al. 2006). Control of any invasive species involves identifying a framework where policy makers, managers, researchers and other stakeholders can establish a comprehensive way to control and

monitor pernicious species (Bax et al. 2001). Population control may involve biological (directed use of predators, sterilisation), mechanical (trapping) or chemical (biocides) methods.

Currently there are no effective methods for the eradication of unwanted populations of non-indigenous crayfish. Efforts to reduce the spread of invasive crayfish are hindered as individual females of invasive species are highly fecund, resulting in only a small number of crayfish being required to facilitate population recovery/establishment; therefore eradication techniques need to provide 100% mortality (Peay & Hiley 2001). The main method which may provide a realistic potential to eradicate unwanted populations is the use of biocides, such as natural pyrethrum (Peay et al. 2006). However the application of biocides can be hard to contain and therefore is only considered appropriate where populations are threatening refuge populations of native species (Hiley 2003a).

Invasive crayfish can be problematic when they occur in high densities and therefore although control measures may not provide a long-term solution, efforts could help to reduce numbers and maintain stakeholder co-operation. Current research and methodology have focused on reducing populations through mechanical control (trapping) and/or biological control (sterilisation, fish predators).

Initial research has shown that sterilisation of male crayfish is possible using ionisation radiation (20Gy X rays), however, only a 43% level of sterilisation was achieved (Aquiloni 2008). Although this degree of sterilisation would potentially help to reduce population numbers, density-dependent processes may occur which would result in increases to the reproduction and growth of surviving individuals. Sterilisation methodology therefore requires further investigation.

Long term studies of trapping regimes have indicated that intensive removal of crayfish may help to limit population growth and control nuisance populations (pers. comm. Abigail Stancliffe-Vaughan, Brecks Project, Suffolk), especially when coupled with effective management of crayfish predator populations (Hein et al. 2006; Hein et al. 2007). Improvements to trap design and bait attractiveness could improve efficacy. Mass trapping currently uses food baits, however success can be limited in habitats which provide a natural abundance of food and shelter, decreasing trap attraction (Hiley

2003b). Therefore the use of pheromones has been proposed as an alternative bait, which would hopefully be species-specific and improve efficacy of trapping during the breeding season (Corkum 2004; Stebbing et al. 2004). Pheromone baited traps could therefore improve the ability to detect low density crayfish populations, which currently can go undetected following initial invasion (Hiley 2003b).

Stebbing et al. (2003b) conducted field trials using female conditioned water as pheromone bait and compared catches to food bait, alarm pheromone bait mixed with food and a blank control. Alarm pheromone baits proved inconclusive in acting like a trapping deterrent. However, traps baited with sex pheromones caught a comparable number of male crayfish over breeding season but were not as effective as food baits at other times, and were ineffective at catching female crayfish. Whether traps baited with sex pheromones will be more effective than food baited trap still lacks a definitive answer.

Further research is required as to how crayfish utilise chemical signals and whether attractant pheromones play a key role in their mating system. Effective pheromone traps require the target species to show far field attraction to the chemical signal but ideally will not attract other species. Development of a suitable trap therefore requires a comprehensive knowledge of the chemical communication system of the target species and closely related species. The identification of the specific chemical components of the pheromone, which will be deployed as bait, is also necessary prior to the mass production of traps. At present the chemical composition of crayfish pheromones is unknown and few studies have investigated the specific behavioural function of chemical cues. This has resulted in the development of weak bioassays which are not be suitable for use in bioassay driven purification techniques used to identify pheromone components.

1.5 Current knowledge of the role of chemical communication in crayfish, in the context of behaviour

Crayfish utilise chemicals to regulate a wide range of social interactions, including reproduction (Ameyaw-Akumfi & Hazlett 1975; Stebbing et al. 2003a), formation of dominance hierarchies (Zulandt Schneider et al. 2001), predator avoidance (Blake & Hart 1993), foraging (Corotto & O'Brien 2002) and inter-specific communication (Tierney & Dunham 1982; Tierney & Dunham 1984). Crayfish chemical

communication studies have predominantly focused on the role of pheromones during reproduction and agonistic activities.

Chemical signals in crayfish are urine-borne, with the release of urine increasing during agonistic (Breithaupt & Eger 2002) and reproductive (Simon & Moore 2007) behaviours. Stressed crayfish have been found to produce significantly more urine than non-stressed crayfish and this signal causes conspecifics to move away from the source (Zulandt Schneider & Moore 2000).

1.5.1 Inter-specific chemical communication

Inter-specific communication can be an important reproductive isolation mechanism in sympatric species. Previous studies have shown *Orconectes* species can chemically recognise conspecifics with similar ranges (Tierney & Dunham 1982; Tierney & Dunham 1984). Sex-specific differences in inter-specific communication have been found in *O. virilis* as male crayfish have been found to distinguish conspecifics from heterospecifics where females could not (Hazlett 1985).

Invasive crayfish species bring into question the effectiveness of interspecies communication as a way of preventing reproductive interference. Male *P. leniusculus* have been found to mate with smaller A. *astacus* and *A. pallipes* (Holdich & Domaniewski 1995; Westman et al. 2002). The chemical presence of male or female *P. leniusculus* repels *A. pallipes* of both sexes, however *P. leniusculus* are attracted to water conditioned by female *A. pallipes* within the breeding season (Stebbing et al. 2006).

1.5.2 Reproduction

Unlike many crustaceans (lobsters, crabs), crayfish are intermoult breeders and as such mating is not tied to the female moult cycle. Crayfish live a solitary lifestyle although males switch from territorial to nomadic behaviours and display increased intra-sexual aggression when trying to locate a mate (Gherardi et al. 1998).

Crayfish follow a characteristic behavioural pattern during mating (Mason 1970; Ingle & Thomas 1974; Villanelli & Gherardi 1998; Stebbing et al. 2003a). Observations of pre-mating activity have led to the suggestion that animals meet at random and sex discrimination occurs during pre-copulatory aggression (Mason 1970; Ingle & Thomas

1974). Mating is generally proceeded by fights, where the female can resist mating attempts (Gherardi 2002). During pre-copulatory aggression male crayfish seize the female by the rostrum or antennae and use the chela(e) and pereopods to mount and turn the female. Females can aid male mounting attempts by becoming immobile, with their claws stretched infront of their body (Villanelli & Gherardi 1998; Gherardi 2002; Stebbing et al. 2003a). Reproduction is via external fertilisation where spermatophores are deposited on the ventral surface of the female, or, in cambarids, into a cornified seminal receptacle, the *annulus ventralis* (figure 1.1B). Males have not been found to show extensive mate guarding of females (pers. obs.; Villanelli & Gherardi 1998; Rubolini et al. 2007), which is common with species that moult prior to mating (e.g. shore crab, *C. maenas*).

Female crayfish spawn shortly after copulation, with mature eggs being released through the gonopores where the spermatophores are dissolved and the eggs are fertilised (Reynolds 2002). The eggs are attached to the pleopods for brooding using glair from the glair gland as an adhesive (Mason 1970). Females invest substantially more energy reserves and time into rearing their offspring than their male partners. Female signal crayfish spawn in the autumn following copulation and eggs are guarded throughout the winter, until they hatch in the subsequent spring. During this time females minimise feeding and continuously fan and groom the eggs, restricting predator escape responses (Reynolds 2002). Aggressive interactions involving berried females can lead to egg loss (Woodcock & Reynolds 1988).

Eggs hatch into motionless juveniles, still attached to the mother's pleopods and require two/three successive moults before they resemble the mobile adult form (Reynolds 2002). Juveniles can stay with females for several weeks, under the control of a maternal brood pheromone, which is released after egg deposition (Little 1976). The pheromone may have a dual role of allowing young to distinguish the mother from other brooding adults whilst repelling other adult crayfish, preventing cannibalism. The chemical is most effective when the eggs hatch and ceases when larvae are fully independent and the mother returns to being cannibalistic.

In contrast to female crayfish, who provide sole parental care for up to six months, males do not invest in mate guarding and are able to inseminate multiple females (Villanelli & Gherardi 1998). The large asymmetry in investment suggests that sexual

conflict may be particularly strong in crayfish, with females only wishing to mate with the best quality male, whilst males try and maximise the number of potential mates (Trivers 1972; Parker 1979) For mating systems which co-ordinate pair formation through visual and acoustic signals the male takes on the costly and risky role of signalling whilst the female searches for a partner (Andersson 1994). However, studies of chemical communication systems, such as in moths, have indicated females release attractive pheromones which elicit male searching and/or reproductive behaviours (Phelan 1997).

Previous studies have yielded contrasting results as to the importance of a female urinary sex pheromone in male recognition of female receptivity (Ameyaw-Akumfi & Hazlett 1975; Itagaki & Thorp 1981; Dunham & Oh 1992; Stebbing et al. 2003a). Recent studies using the dye fluorescein as a visual marker for urine release have indicated that pheromones may play an important role in reproduction. Reproductively active crayfish (*O. rusticus*) were found to release significantly more urine than crayfish which were non reproductive (Form II male, female non-glair) (Simon & Moore 2007). However these results did not look at urine release during mating behaviours as only a low percentage of trials (38%) between reproductively active crayfish resulted in any mating attempts. The behavioural function of the female sex pheromone is therefore still unknown. Considering the apparent contradiction between females showing resistance to mating during precopulatory aggression and the evidence for a female sex pheromone the major question remaining to be answered is do females release a pheromone to attract male partners and elicit sexual behaviours?

In order to study the chemical composition of sex pheromones a suitable bioassay needs to be developed to drive purification techniques. Previous studies have focused on inter-sex differences in posture and chemotactic abilities when exposed to odours from conspecifics; i.e. male crayfish display submissive behaviours to female odour and agonistic postures to male odour (Ameyaw-Akumfi & Hazlett 1975). However these kinds of studies do not provide a basis for inferring the behavioural function of the pheromone. Displays of specific courtship behaviours in the presence of female odours would provide reliable evidence of a female sex pheromone and form the basis of a reliable bioassay. Stebbing et al. (2003a) devised a bioassay where female urine or conditioned water was released through an air-stone into a tank containing a male *P. leniusculus*. During two trials male crayfish deposited spermatophores on the air-stone

which is a specific courtship response to the chemical cue. However this behaviour was not reliable and repeatable and subsequently the devised bioassay was based on ambiguous behaviours (increased movement and handling of the air-stone). In addition, responses were not tested for sex specificity. Therefore this assay is unlikely to be successfully employed in bioassay-driven purification of the pheromone.

1.5.3 Mate choice

Due to the high investment in gamete production and rearing their offspring, female crayfish need to choose good quality partners. Female crayfish have been shown to choose large male partners with large, symmetrical, chelipeds (Villanelli & Gherardi 1998; Gherardi et al. 2006; Aquiloni & Gherardi 2008c). However it is unclear whether females can distinguish the quality of male partners through chemical cues. Y-maze choice tests comparing female preference of odours from dominant and subordinate male crayfish have remained inconclusive (Zulandt Schneider et al. 1999) and females could not distinguish dominant males when choosing from size-matched individuals (Villanelli & Gherardi 1998). However, if females can observe male fights they are capable of choosing the dominant opponent, when given a free choice (Aquiloni et al. 2008). When eavesdropping on male fights, females received both visual and chemical cues, therefore it is still uncertain which stimuli was of higher importance when choosing a male partner.

In comparison to females, male crayfish have larger chelae in relation to their carapace length and are therefore capable of overpowering their sexual partners through sexual coercion. Large male crayfish have the competitive advantage and have increased access to resources in comparison to smaller, subordinate opponents. Therefore, non-random mating may be common in populations, with large males having the competitive advantage over smaller, subordinate opponents (Gherardi et al. 2006). Due to the potential for sexual coercion in crayfish females can choose to exert a post-mate choice and adjust egg and clutch size according to male traits (Galeotti et al. 2006). The size and weight of the egg clutch and resultant juveniles are larger when sired by larger fathers (Aquiloni & Gherardi 2008b).

In some mating systems, where males produce spermatophores, males can exhibit mate choice of female partners. This phenomenon has been attributed to the relatively high cost of spermatophores (Dewsbury 1982). There appears to be no experimental evidence

for male choice of female partners in crayfish, as males predominately mate with the first receptive female encountered (Villanelli & Gherardi 1998). However males can modulate sperm allocation depending on female attributes and time in the mating season, with highest expenditure for large females and at the end of the breeding season (Rubolini et al. 2006). Larger males have lower sperm expenditure than smaller males, possibly by economising for multiple matings, or, due to senescence (Rubolini et al. 2006). Crayfish are polygamous with sperm competition in multiple matings, where males may feed on previously deposited spermatophores prior to mating, giving last male advantage (Reynolds et al. 1992; Villanelli & Gherardi 1998).

1.5.4 Agonism

Crayfish are model organisms to study agonistic behaviours as (i) they frequently engage in fights forming dominance hierarchies (ii) vision is easy to segregate and (iii) long term isolation can eliminate the effects of previous interactions (Issa et al. 1999; Goessmann et al. 2000; Zulandt Schneider et al. 2001; Hemsworth et al. 2007).

Agonistic behaviour in crayfish, like most decapods, is highly ritualised, following a strict rule of behavioural patterns which are exhibited in a stereotyped pattern (Atema & Voigt 1995; Huber & Kravitz 1995; Breithaupt & Eger 2002). Fights start with initiation behaviours and threat displays, leading to contact, where the claws grasp and pull at the opponent until use is unrestrained (tearing, ripping). Fights can consist of a number of bouts which ultimately result in one animal retreating, possibly using a tail flip retreat mechanism (Karavanich & Atema 1998a; Bergman et al. 2003). Physical aggression is coupled with a change in physiological state, both heart rate and ventilation rate increases with increasing fight intensity (Schapker et al. 2002).

Individual fighting success can depend on a number of factors such as physical superiority (Figler et al. 1995; Rutherford et al. 1995), social experience (Issa et al. 1999; Goessmann et al. 2000), behavioural strategies (Guiasu & Dunham 1997; Hack 1997), knowledge of resource value (Smith et al. 1994), prior residence effects (Peeke et al. 1995) dietry effects (Vye et al. 1997) and moult stage (Tamm & Cobb 1978). Crayfish form linear dominance hierarchies that are relatively stable with fight intensity and duration decreasing with time (Issa et al. 1999; Goessmann et al. 2000). Dominant males usually initiate fights and show high levels of aggression in comparison to the

sub-ordinate opponent, which retreats at the end of the bout and does not initiate more fighting.

Agonistic bouts between crayfish are mediated by chemical signals released in the urine (Zulandt Schneider et al. 2001; Zulandt Schneider et al. 1999; Breithaupt & Eger 2002). The use of the dye fluorescein as a visual marker for urine release has demonstrated that crayfish increase urinary signalling during social interactions and in fights offensive behaviours are more effective when performed in conjunction with urine release (Breithaupt & Eger 2002).

Choice tests have indicated that male crayfish can recognise the social status of conspecifics through chemoreception (Zulandt Schneider et al. 1999). Unlike lobsters, crayfish dominance cues are based on status specific chemical signals released in the urine, rather than signals of individual identity (Copp 1986; Karavanich & Atema 1998a; Karavanich & Atema 1998b; Zulandt Schneider et al. 2001; Breithaupt & Eger 2002; Gherardi & Daniels 2003). The recognition of status is evident as second fights are of shorter duration than initial fights, irrespective of whether the opponent is familiar or unfamiliar (Breithaupt & Eger 2002).

The chemical identity of urinary status cues in crayfish is currently unknown and it remains to be established whether a single chemical component or a chemical mixture mediates dominance recognition. Previous research has indicated that biogenic amines, such as serotonin, octopamine and dopamine, may play an important role in agonistic interactions and regulating dominance hierarchies (Huber et al. 1997a; Huber et al. 1997b; Sneddon et al. 2000; Tricarico & Gherardi 2007).

Although considerable research effort has focused on developing bioassay methodology to identify crayfish sex pheromones, limited research has been directed at designing a bioassay to identify male status cues. It is important to have a reliable bioassay for sex pheromones and for agonistic pheromones as discovering the chemical identity of these stimuli will provide new and exciting opportunities to quantitatively study the biology of crayfish.

1.6 Aims and Objectives

To date few studies have investigated the specific behavioural function and the natural context of crayfish chemical signalling. An incomplete understanding of crayfish chemical communication has delayed progress in the development of appropriate assays for the identification of dominance and sex pheromones. This needs to be resolved before pheromone trapping can be considered a serious control measure for nuisance populations of invasive crayfish. Previous bioassay designs have relied upon weak behavioural responses (increased movement, handling of an airstone) of male crayfish to introduced female odours (conditioned water, urine) and have not tested for sex-specificity (see Stebbing et al. 2003a). These bioassays are therefore unlikely to be effective in driving purification techniques for identification of female crayfish sex pheromones. In addition, there is currently no suitable assay to aid the identification of dominance pheromones which have been found to play an important role in agonistic interactions (Breithaupt & Eger 2002).

This thesis aims to investigate chemical communication in two invasive crayfish species, *P. leniusculus* (signal crayfish) and *P. clarkii* (red swamp crayfish), during both reproductive and agonistic social behaviours. Studies focused on using behavioural and physiological techniques to investigate the natural context of chemical signalling, incorporating both signal delivery and receiver responses. The results from these studies helped to focus the development of reliable assay methodology and ensure biologically relevant parameters were used. The novel assay designs should aid future studies, and drive analytical chemistry techniques to identify the active components of crayfish sex pheromones and dominance cues. Identification of pheromones would help to unlock information on crayfish signalling behaviour, evolution and neurobiology.

Signal crayfish were predominantly used as the study species as they are highly invasive in the UK and therefore easier to source than red swamp crayfish, which are highly invasive though-out the rest Europe.

The aims and major questions of each chapter are described below:

Urinary signalling in crayfish social interactions: miscommunication between females and males?

Dye visualisation techniques were used to investigate the timing and behavioural function of urine release in different social interactions (reproductive behaviours, fighting). Is urine release linked to specific reproductive behaviours? Are there any differences between the signalling behaviour of male and female crayfish and does this reflect any sexual conflict related to mating? Do females release a signal which specifically advertises receptivity?

Mate choice in crayfish, *Pacifastacus leniusculus:* Do females choose dominant partners when given a free choice?

Can females distinguish between the quality of size matched male crayfish through chemical cues? Female crayfish were given a free choice of male partners by tethering males to prevent potential interference from male-male competition. Do females actively choose dominant male partners?

Ventilatory and heartbeat activity of crayfish, *Pacifastacus leniusculus*, during reproductive behaviours

The aim of this study was to assess if consistent changes in physiological parameters of male and female crayfish are detected during reproductive behaviours. This will indicate whether ventilation and heart rate measurements could be used in an assay to help identify crayfish sex pheromones.

Ventilatory measures in crayfish during social interactions: the effects of social dominance and urinary signalling

Do crayfish actively increase ventilatory activity during the release of urinary signals in agonistic encounters? Is this a form of multimodal signalling? How does ventilatory activity change during agonistic interactions and is ventilation rate a suitable parameter for a bioassay to measure male responses to agonistic pheromones?

Development of behavioural and physiological assays to assess discrimination of male and female odours in crayfish, *Pacifastacus leniusculus*

The development of a reliable, biologically relevant assay is essential when coupling analytical chemistry techniques with bioassay driven purification. The aim of this study was to investigate whether behavioural or physiological measures can form the basis of a reliable and reproducible assay to determine if male crayfish can distinguish odours (urine, conditioned water) of male and female crayfish.

The effects of artificial urine release on the fight dynamics of crayfish, *Pacifastacus leniusculus*

Can dominance recognition be established in second day fights by artificially introducing urine from the dominant animals in fight one? Does urine from the dominant male cause the loser of the initial fight to give up early in the second fight and display lower levels of aggression? Could the methodology of blocking urine and artificial urine introduction be developed into a bioassay for chemical identification of dominance cues?

Chapter 2

General Methodology

2.1 Animal Maintenance

2.1.2 Collection

Throughout this research signal crayfish were obtained from a crayfish dealer, Chris Campbell, North Dorset. Crayfish were caught in a local lake (Whistley Waters, Milton on Stour, Gillingham, Dorset) using Swedish Trappy Traps baited with high protein fish pellets.

Red swamp crayfish were obtained from crayfish suppliers, Sunbeam Aquarium Ltd (Singapore). Crayfish were caught in China and transported to the U.K. through GAC Logistics (Manchester), in line with the DOF 7 import licence from DEFRA (Department for Environment, Food and Rural Affairs).

All animals were allowed to acclimate to laboratory conditions and recover from handling and transportation stress for one - two weeks prior to experimental use. New animals were obtained at the start of each experimental series to minimise the possible effects of laboratory conditions of behaviour.

2.1.2 Legislation and holding

Both signal crayfish and red swamp crayfish are prevalent invasive species and therefore relevant licences and caution was required when maintaining live stocks. Under the Prohibition of Keeping Live Fish (crayfish) Order 1996 a licence from DEFRA was obtained to keep *Pacifastacus leniusculus* and *Procambarus clarkii* (Licence holder: Dr. Thomas Breithaupt). Animals were maintained in self contained aquaria, where necessary precautions were taken to prevent escapees; all tanks were fitted with removable lids and the main drainage of the holding room was covered with a grid filter (1 mm pore diameter), therefore even if juveniles were kept they could not escape. The facilities were monitored for diseases under EC guidelines by the CEFAS (Centre for Environment, Fisheries and Aquaculture Science) fish health inspectorate.

Crayfish were kept in two holding systems. Both aquaria comprised of 6 tanks (45 x 60 x 30cm) in a recirculating freshwater system containing carbon filters (Pozzani Pure Water PLC, UK), and an additional UV filter (Lotus Water Garden Products, UK). To supplement the oxygen supply, each tank had air supplied to it through an airstone. Coral sand covered the bottom of each tank to maintain calcium levels, which is

important when animals are moulting. Animals were separated by sex and isolated in separate tanks.

When signal crayfish and red swamp crayfish were housed simultaneously they were kept in separate holding systems. To keep red swamp crayfish isolated from each other, a divider was created which split the tank into 15 compartments (1 cm x 1 cm holes in the divider to allow water movement between compartments). This prevented animals from fighting, which has previously been found to result in limb autotomy (Dr. T. Breithaupt, pers. comm.). Signal crayfish displayed lower levels of aggression when housed communally and were kept at a maximum density of 25 individuals per tank. Tanks contained broken plant pots which acted as shelters, further reducing agonistic encounters.

Aquaria were maintained in a temperature and light controlled room to reflect conditions found in the natural environment. Signal crayfish were kept in conditions which mimicked the ambient seasonality of temperatures and light conditions of streams in the UK. Red swamp crayfish were maintained in a constantly warm environment (21°C) with 14: 10 light: dark lighting regime.

All animals were fed twice a week on commercially available, defrosted prawns.

2.2 Experimental procedures

2.2.1 Identification

Crayfish were identified by numbering individuals using white corrective fluid. Animals were numbered on both the left and right side of the carapace. Marked crayfish were washed thoroughly and no side effects were found from this tagging method. Animals remained numbered until they moulted, which was sufficient for experimental purposes.

2.2.2 Isolation

Prior to all experiments crayfish were isolated for one - two weeks in separate 3 litre PVC containers (24 x 17.5 x 8 cm). Isolation should limit the effects of prior social encounters on the outcome of experimental interactions (Zulandt Schneider et al. 2001; Hemsworth et al. 2007).

2.2.3 Blindfolding

Reversible blindfolding of crayfish was necessary to prevent potential behavioural reactions to visual disturbances during the experimental period. Crayfish were blindfolded 24 hr prior to experiments by wrapping opaque plastic (1cm x 4cm) around the rostrum and eyestalks and securing excess material to the carapace using cyanoacrylate glue (figure 2.1). When blindfolded animals displayed the same range of behaviours as observed when animals were not visually impaired (pers. obs.). After the experiment blindfolds could be removed without damaging the eyes or the carapace.

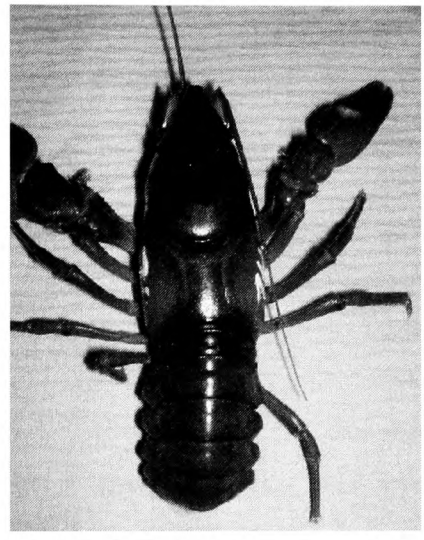

Figure 2.1: A blindfolded signal crayfish (*P. leniusculus*). A strip of black opaque plastic was wrapped around the rostrum and secured to the dorsal carapace using cyanoacrylate glue.

2.2.4 General experimental protocols

All animals were acclimatised to experimental tank environments for at least 10 min. This ensured experiments were not biased by crayfish displaying potential stress/investigatory behaviours in response to being in a novel situation. An opaque PVC divider was used to physically and chemically separate animals during acclimatisation periods.

2.2.5 Checking for receptivity

Although signal crayfish have a relatively short breeding season (late Sept – Nov) individuals may not be receptive over the whole season. It was therefore important to ensure that animals were receptive prior to performing experiments on reproductive behaviours. Following an acclimatisation period of 10 min, behaviour of a male and female adult crayfish was observed for 20 min in a glass aquarium (30 x 20 x 20 cm).

Males were classed as sexually receptive if they tried to turn or mount the female, whereas receptive females showed a 'receptive posture'; the claws are outstretched in front of their body, which is lowered towards the substrate, aiding the male in mounting. In addition, female crayfish were identified as sexually receptive if glair glands were visible as whitened tissue on the underside of the telson. Glair acts as a protective substance when eggs are released and assists egg attachment to the swimmerets (Reynolds 2002).

Unreceptive animals were returned to the holding aquaria and were not used in experiments investigating sexual behaviours.

2.2.6 Urine visualisation

Fluorescein was used to visualise urine release following the methods developed by Breithaupt & Eger (2002). A 0.3 % sodium fluorescein solution (dose 9 – 10 μg g^{-1} body mass) was injected into the pericardium region of crayfish three/four hours prior to experiments using a 250 μl syringe (Hamilton, Switzerland) and a short needle (0.45 x 13mm, Microlance, Ireland). After injection the carapace hole was sealed using plasticine and tape to avoid haemolymph loss. Following injection crayfish were returned to isolation and fed on defrosted prawns.

After the experiment if there had been no visible signs of urine release from an individual, the animal was left until spontaneous urine was released to ensure that the visualisation procedure was successful.

Post-experimentation all animals were checked for mortality. Mortality was infrequent and generally occurred following a relatively high dosage of fluorescein in a small crayfish (N = 4). For this reason animals weighing < 20 g were excluded from urine visualisation experiments. Throughout the course of this thesis approximately 200 animals were injected with fluorescein, without negative side effects.

When filming urine release interactions took place in a glass aquarium (40 x 20 x 20 cm) which was adapted to provide increased contrast by covering the walls with black opaque lining. Light from a 150 W slide projector (Reflecta Diamator AF, Germany) was reflected into the tank by a mirror (44 x 20 cm). The projector provided the bright light necessary to film fluorescein release.

2.2.7 Urine sampling technique

In order to investigate the potency of urinary signals to stimulate responses in conspecifics urine samples were collected from live animals. The direct urine sampling technique was a non-lethal and efficient procedure.

Crayfish were strapped to a small plastic board, using elastic bands. This ensured the animals were stationary and the ventral surface was exposed. The third maxillipeds were moved away from the nephropores (the excretion pores) and secured with elastic bands. Small pieces of absorbent paper were inserted into the gill openings to ensure any water exuded from the gills was not sampled with the urine. A micropipette tip (1-10 µl, Fisherbrand) was inserted into the opening of the nephropore which caused urine to flow out from the bladder, to be collected by suction into Teflon tubing (1.5 mm diameter) and a 1.5 ml collection vial (modified from Bamber & Naylor 1997). This process was repeated for both nephropore openings. Approximately 50 µl to as much as 1 ml could be collected from each individual crayfish. Urine was either used directly in experiments (less than one hr post collection) or was immediately transferred to the freezer for overnight storage at -25°C (all urine was used within 24 hr of collection). Between sampling equipment was washed fully using distilled water. A new micropipette tip was used for every individual.

2.2.8 Conditioned water

Water was conditioned by keeping individual crayfish for 24 hrs in a plastic container (12 x 12 x 6 cm) filled with 300 ml filtered water, (filtered through a 25 cm pre-sediment filter followed by a 25 cm activated carbon filter, Pozzani Pure Water PLC, UK). Water was conditioned by an individual crayfish, instead of multiple animals like in previous studies (Stebbing et al. 2003a), to reduce the chance of stress or agonistic pheromones being released into the water.

2.2.9 Urine Blocking

Urine was blocked using a non-lethal reversible technique. One cm of silicon tubing (1.6mm diameter, Bio-Rad, USA) was attached to the basal segment of the second antenna around the nephropore, using cyanoacrylate glue (Zap-a-Gap, Pacer). To prevent leakage an additional cyanoacrylate layer was applied to the tube and dried using an accelerator fluid (Zip kicker, Pacer). Plasticine plugs were inserted into the end

of both tubes. Dye studies were conducted to ensure that the plasticine plug was efficient at blocking urine release from fighting crayfish. Fluorescein dye was used as a marker for urine release by injecting fluorescein into the heart region of adult male crayfish at a dosage of 10 μg/g. The silicon tubing and plasticine plug was efficient at preventing urine release in all animals tested (N = 6), with urine visible in the plugs of five individuals.

2.2.10 Behavioural analysis

Behavioural analysis was performed by digitising filmed experiments and using a behavioural software package (The Observer 5.0, Noldus Information Technology, Wageningen, The Netherlands) to score behaviours. Different behaviours could be scored throughout the experiment on a continuous time scale.

2.2.11 Physiological Measurements

2.2.11.1 *Heartbeat analysis*

Heartbeats were monitored using a non-invasive optoelectronic technique (figure 2.2; Depledge & Andersen 1990). An infrared sensor (figure 2.3; 12 mm diameter; A312-95 optosensor IR, Farnell, USA) was attached to the carapace over the cardiac region of the crayfish using cyanoacrylate glue. The sensor is comprised of a light emitting diode (wavelength 950 nm) and a phototransistor detector. The phototransistor could detect the changes in infrared light intensity scattered as a result of the heart beating. The sensor was connected to a two channel amplifier, which filtered and amplified the signal (Dept. of Engineering University of Hull). The amplified signal was transferred to a computer using a PowerLab AD converter (type ML880, ADInstruments).

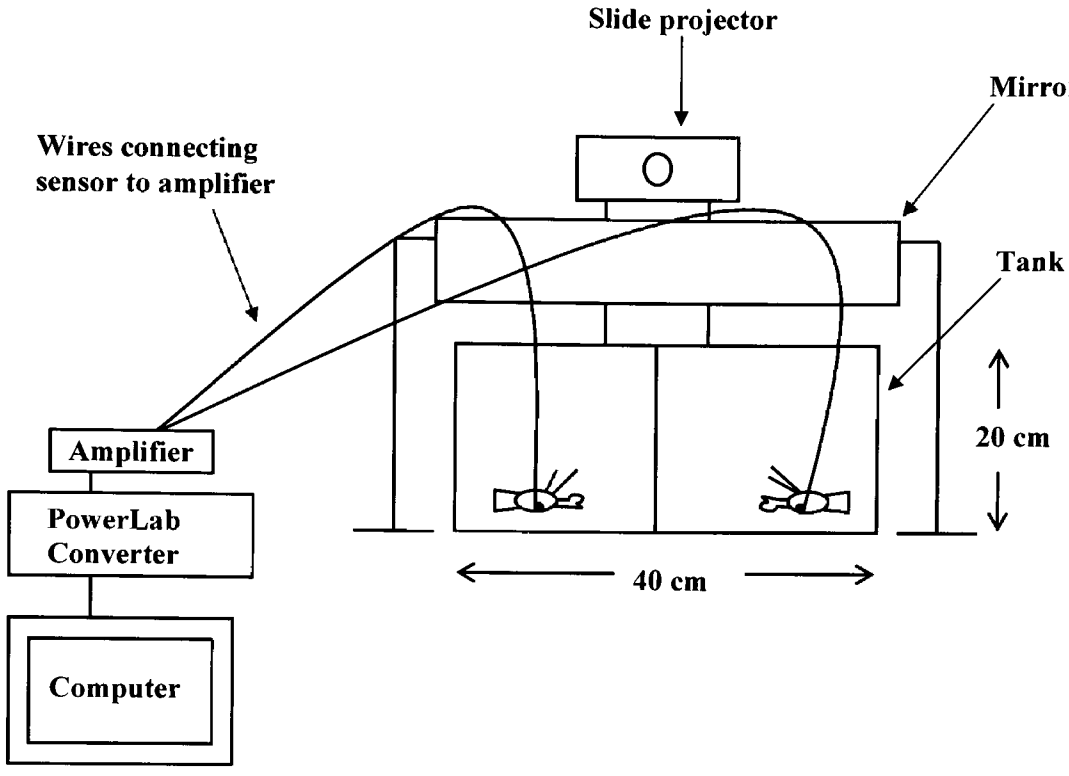

Figure 2.2: Schematic diagram of the experimental equipment used to measure physiological parameters (ventilation rate, heart rate) of crayfish. An infrared sensor was attached to the carapace (a) above the anterior of the right branchial chamber to record ventilation rate and (b) to the carapace above the heart region to record heart rate. Light from a slide projector was reflected into the tank via a mirror. Wires from the sensors were hung over the mirror, which helped to prevent wire entanglement during experiments.

Heartbeats were measured in mV and analysed using Chart 5.5 software (ADInstruments) which calculated an instantaneous heart rate (BPM, beats per min) and the periods (sec) between individual heartbeats.

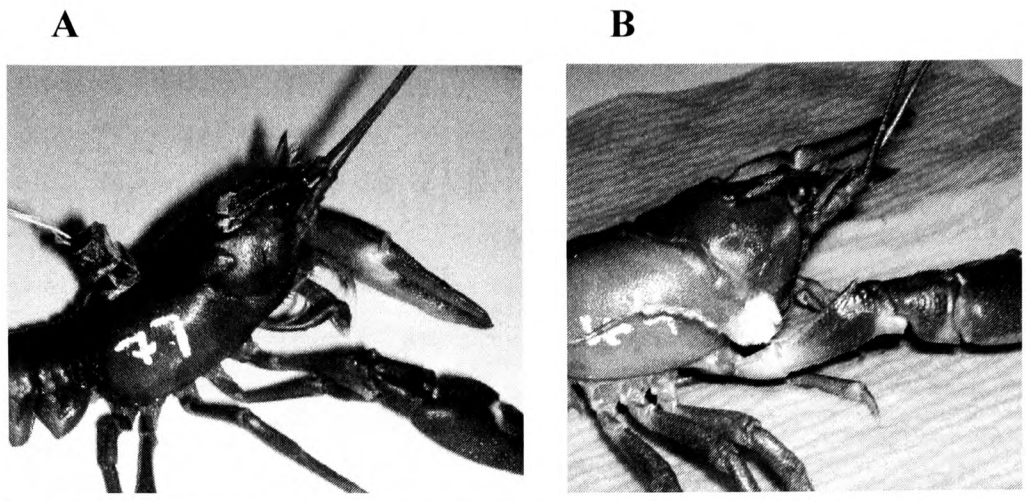

Figure 2.3: Examples of the infra-red sensors used to measure (A) heartbeat activity (B) scaphognathite movement of signal crayfish. Sensors are attached to the carapace over the cardiac region (heartbeat analysis) or to the anterior of the right branchial chamber to detect scaphognathite beat activity. Sensors to measure ventilatory activity were smaller than heartbeat sensors, to reduce hindrance to cheliped movement.

2.2.11.2 *Scaphognathite beat analysis*

The creation and maintenance of water currents through the branchial chamber of crayfish is achieved by the beating of a pair of scaphognathites. Scaphognathites are enlarged plate-like exopodites of the second maxillae which lie in the narrow channels to the anterior of the left and right branchial chambers. Beating of the scaphognathite (in a seesaw like motion around a middle hinge) results in the forward projection of water and simultaneous creation of negative pressure in the branchial chamber, causing water to enter through inhalant channels between the limb bases (Vogt 2002). Therefore measurement of scaphognathite movement gives an indication of the ventilation rate of crayfish.

The ventilation rate of crayfish was recorded by measuring scaphognathite beats, using the same non-invasive optoelectronic technique as used for heartbeat analysis (figure 2.2; Depledge & Andersen 1990). The infrared sensor used to measure ventilation rate was smaller than that for heartbeat analysis (figure 2.3; 4 mm diameter, SG-2BC, optosensor IR, Farnell, USA), this reduced the potential impedance of chelae movement by the sensor. The sensor was attached to the carapace above the anterior of the branchial chamber. Scaphognathite beats were measured in mV and analysed using Chart 5.5 software (ADInstruments), which calculated an instantaneous rate (BPM, beats per min) and the periods (sec) between individual beats.

In order to reduce the level of handling and potential hindrance of chelae movement by the infrared sensors, measurements of ventilation rate were only recorded for the right branchial chamber. Previous studies of crustaceans have indicated that pairs of scaphognathites can be capable of independent activity but in general show absolute rates that are similar (Cumberlidge & Uglow 1977). Preliminary studies were carried out to assess whether the left and right scaphognathites of crayfish beat with the same frequency. Ventilation rate was measured for both branchial chambers whilst animals were isolated and was recorded for ten female signal crayfish, ten male signal crayfish, ten female red swamp crayfish and ten male red swamp crayfish. Absolute rates were, in general, very similar between each scaphognathite but were not identical (figure 2.4). The ventilatory activity left and right scaphognathites were compared over a one minute time-frame. This showed the maximum difference in rate between sides was 10 %, with a mean difference of 2.4 % ± 2.27 (male signal crayfish); 3.9 % ± 1.04 (female signal crayfish); 6.3 % ± 0.84 (male red swamp crayfish); 5.3 % ± 1.27 (female red swamp crayfish). None of these differences were found to be significant (Paired t tests; $p > 0.05$).

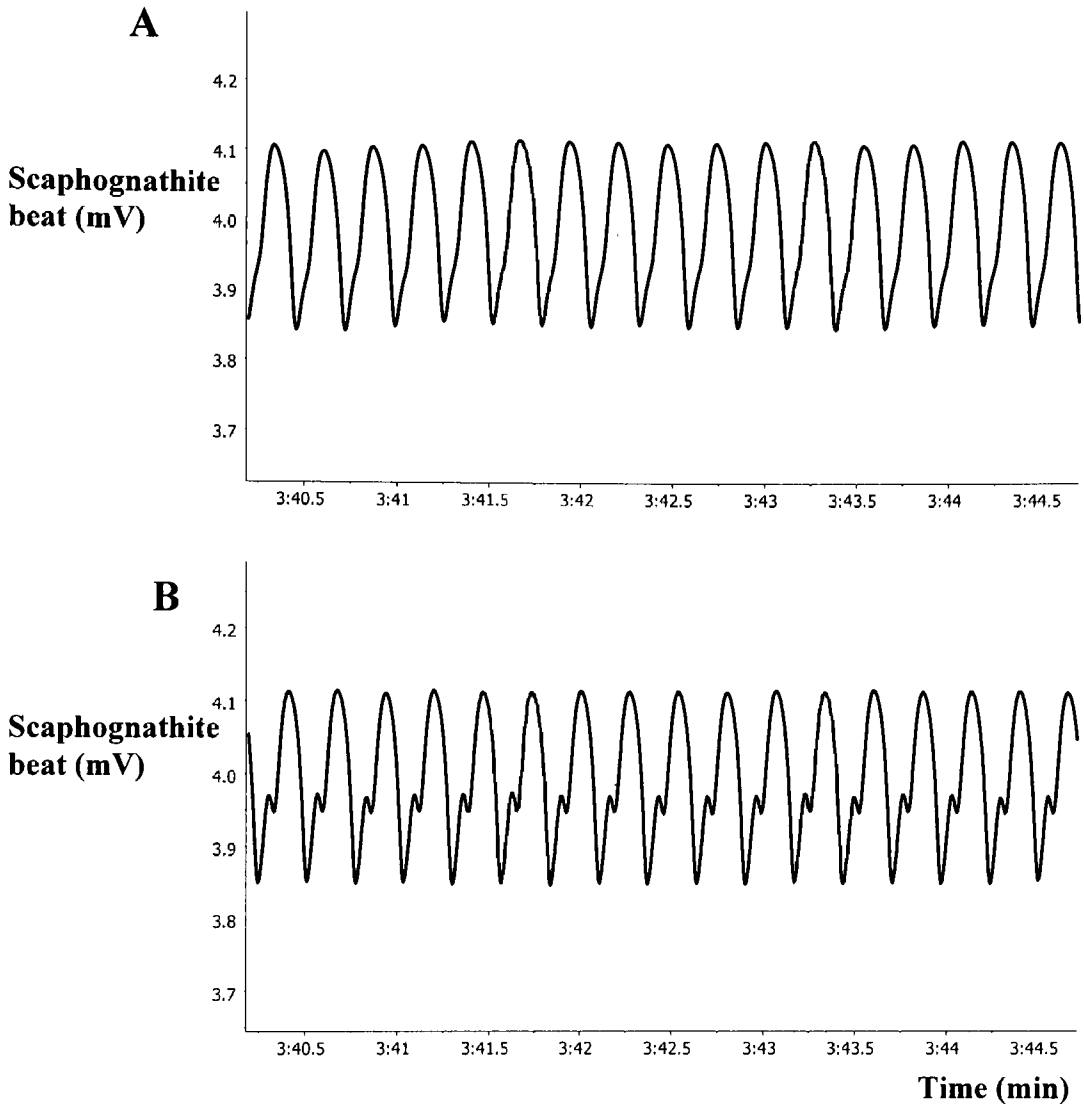

Figure 2.4: Ventilation rate recordings of an isolated male signal crayfish; (A) right scaphognathite recording (B) left scaphognathite recording. Scaphognathite recordings are not identical for both sides however the overall ventilation rate of this section is comparable (at 225 beats per min).

In addition to testing ventilation rate in isolated animals, the ventilation rate was recorded for both scaphognathites whilst two males were engaged in aggressive fighting. Again, scaphognathites were found to beat at a similar rate and when abrupt changes in ventilation rate occurred as a result of agonistic encounters, the change appeared to be bilaterally synchronized (figure 2.5).

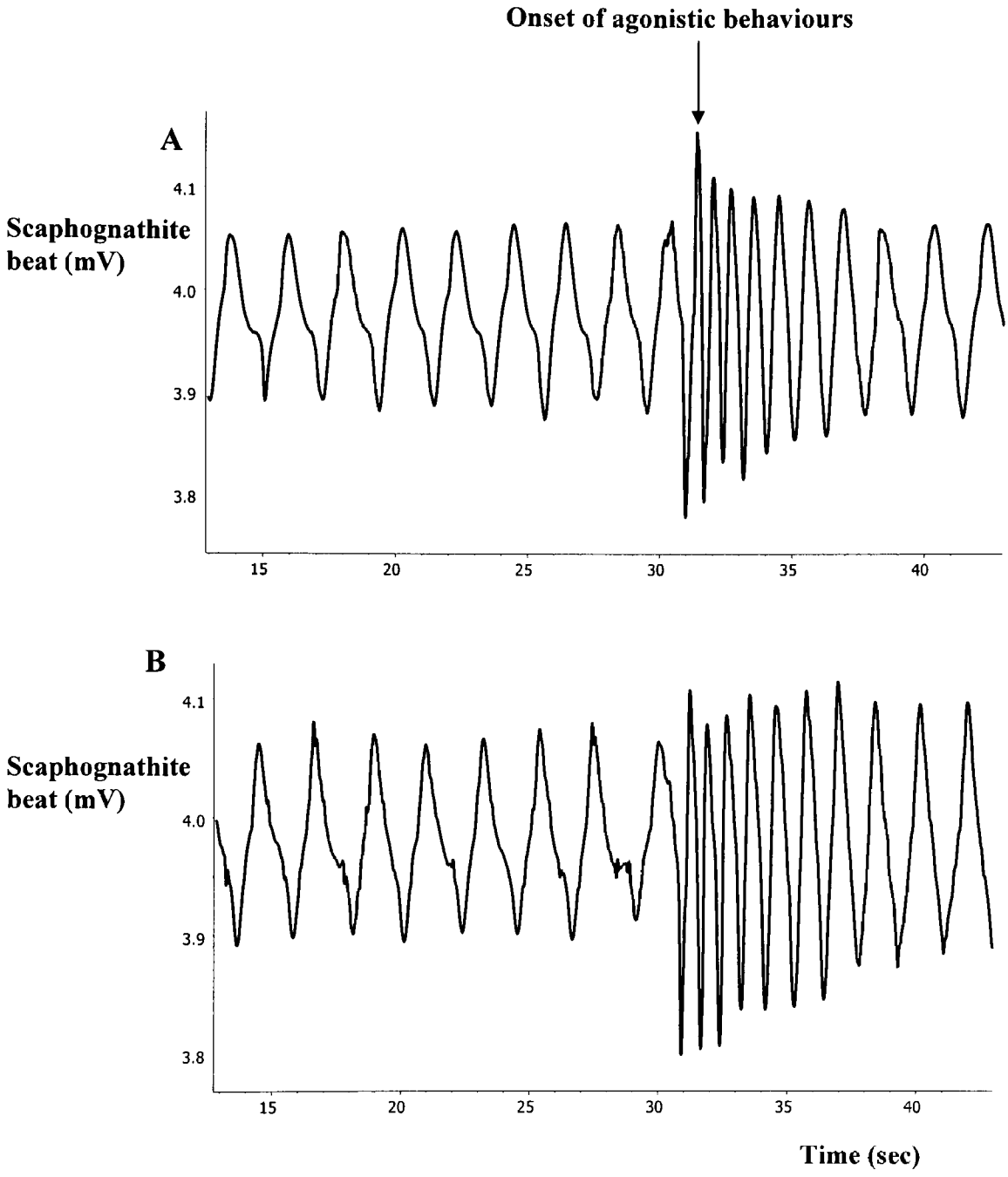

Figure 2.5: Ventilation rate recordings of a male red swamp crayfish which was engaged in fighting; (A) right scaphognathite recording (B) left scaphognathite recording. Scaphognathite recordings are not identical however at the onset of aggressive behaviour there is bilateral change in ventilation rate.

It was concluded that for future studies scaphognathite movements would only be measured for one branchial chamber. To standardise between subjects and experiments an infrared sensor was attached to the anterior of the right branchial chamber only. Logistically it was also advantageous to measure beats of only one scaphognathite as during the preliminary fight trials with bilateral sensors there was a high tendency for

opponents to become tangled in the wires connecting the infrared sensor to the amplification box.

2.2.12 Statistical analysis

All statistical analysis was performed using the statistical packages JMP IN v5.1 (SAS Institiute Inc, Cary, USA) and SPSS v 13.0 (SPSS Inc, Chicago, USA).

Chapter 3

Urinary signalling in crayfish social interactions: miscommunication between females and males

3.1 Introduction

Sexual selection theory predicts that females will minimise risk and energy expenditure during courtship, due to the higher investment in their offspring than male partners (Andersson 1994). Asymmetry in the evolutionary interests of males and females can result in sexual conflicts over whether a mating takes place (Parker 1979). Males try to maximise the number of mating opportunities whilst females are highly selective in choosing when to mate and the quality of their partner (Trivers 1972). In general males therefore perform the more costly role in pair-formation and invest in courtship signals to compete for mating opportunities with the choosier sex. Previous research has primarily focused on the role of sexual selection in shaping acoustic and visual advertisement signals with limited effort directed at the role of chemical signalling in courtship behaviour (Andersson 1994).

Chemical communication systems, such as in moths (Alexander et al. 1997) and decapod crustaceans (Atema & Steinbach 2007), often involve a female signaller and male receivers. Females appear to initiate courtship through the release of a sex pheromone which triggers the male's mate search and/or courtship behaviours. For example, female shore crabs (*Carcinus maenas*) release a pheromone which elicits male mating behaviours: when exposed to female urine males have been found to attempt to mate with inanimate objects, such as stones or sponges (Hardege et al. 2002). While the male's response has clearly evolved to be effected by the female stimulus, it can be questioned whether the female's release of olfactory stimulants has evolved for the purpose of eliciting this response (Williams 1992). Modern definitions of communication emphasise that both signal and response need to be adaptive (Maynard Smith & Harper 2003; Scott-Phillips 2008). Williams' questioned the existence of female pheromones, such as those produced by moths, based on the fact that they are 'produced in minute traces' and 'not by machinery designed by selection to produce a male response' (Williams 1992). His view has been opposed by arguments which favour the evolution of female pheromones (Phelan 1997). For example the release of pheromones by female moths bears a lower predation risk than mate-search by males (Alexander & Borgia 1979) since olfactory receptors of predators (birds, bats) are less developed than their visual/acoustic senses. Furthermore, in insects females release minute traces of pheromones which impose sexual selection on male searchers by facilitating scramble competition where only males with the best searching and chemosensory abilities secure mating opportunities (Greenfield 1981).

These arguments do not apply to female pheromone signalling in aquatic environments. Unlike aerial predators many aquatic predators use chemical cues to detect their prey, increasing the potential risk to a chemical signaller (Zimmer-Faust 1989; Hara 1994). However, evidence from several species of decapod crustaceans suggest that females release urinary signals eliciting specific male responses (Atema & Steinbach 2007). Responses include near-field attraction (Gleeson et al. 1984; Bamber & Naylor 1997) and the release of courtship behaviours (Hardege et al. 2002). In crustaceans, knowledge about the communication route from female to male is predominantly based on the analyses of male responses; little is known about female signal production in relation to courtship behaviours. One exception is a recent study by Simon & Moore (2007) which demonstrates that both male and female crayfish (*Orconectes rusticus*) release urine during sexual interactions.

Crayfish live at high population densities and have multiple opportunities to mate (Gherardi 2002). Females invest considerable energy in rearing their offspring (Reynolds 2002). For example, female signal crayfish (*Pacifastacus leniusculus*) spawn in the autumn and then provide sole parental care for up to six months. Males, in contrast, do not invest in mate guarding and are able to inseminate multiple females (Villanelli & Gherardi 1998). The great imbalance between the sexes in investment into offspring suggests that in crayfish sexual conflict over mating may be particularly strong. Therefore, it is not surprising that mating in crayfish is generally proceeded by fights with females trying to resist male mating attempts (Gherardi 2002). In this scenario, it is expected that females do not invest in courtship signals. However, previous studies have shown that crayfish release urine during sexual interactions and female urine is effective in eliciting male courtship behaviour (Stebbing et al. 2003a; Simon & Moore 2007; Berry & Breithaupt 2008;).

This study aims to address the discrepancy between the experimental evidence for female olfactory courtship signals and the reported resistance of female crayfish to mating. Using the dye fluorescein urine release was visualised in male and female crayfish (*P. leniusculus*) during staged social encounters. The timing of chemical stimulation delivery was analysed in relation to the behavioural context of reproduction versus fighting.

3.2 Materials and methods

3.2.1 Animals

Adult crayfish (*P. leniusculus*) were obtained in September 2005, March 2006 and August 2007 from a crayfish dealer (Chris Campbell, North Dorset), having been trapped in a local lake. This ensured that animals to be used in reproductive interactions were caught prior to the start of the breeding season.

Animals were separated by sex and kept in communal holding tanks (45 x 60 x 30 cm) in a recirculating freshwater system containing carbon filters (Pozzani Pure Water PLC, UK), and an additional UV filter (Lotus Water Garden, UK). A maximum of 25 animals were kept in the tank and males were physically but not chemically separated from females. During the breeding season (Sept – Dec) crayfish were maintained at 10 °C, 10:14 h light:dark cycle, whilst outside of the breeding season (Mar, Aug) crayfish were maintained at 14 °C and 12:12 h.

Within the breeding season, individuals were tested for sexual receptivity. Following an acclimation period of 10 min, crayfish pairs were observed for 20 min (glass tank 30 x 20 x 20 cm). Males were classed as sexually receptive if they tried to turn or mount the female. In addition, female crayfish were identified as sexually receptive if glair glands were visible as whitened tissue on the underside of the telson Unreceptive crayfish which were not used in sexual interactions were returned to holding tanks or used in fight trials.

One week prior to interactions individual crayfish were isolated in separate 3 l plastic containers (24 x 17.5 x 8 cm). Animals were blindfolded 24 hrs prior to the experiment by wrapping opaque plastic (1 x 4 cm) around the eyestalks and rostrum and securing excess material to the carapace using cyanoacrylate glue.

3.2.2 Urinary signalling in social interactions

To study social interactions were used 60 intermoult female crayfish (mean ± SE carapace size of 34.7 ± 0.2 mm, mass 29.1 ± 0.6 g) and 60 intermoult male crayfish (mean ± SE carapace size 36.3 ± 0.3 mm, mass 33.8 ± 0.9 g), with intact appendages.

Male fights, female fights and reproductive behaviours were studied within the breeding season (Oct – Dec) whereas male-female fights were observed out of the breeding season (Mar, Aug). Crayfish were size-matched to eliminate the effects of body size on social interactions. In same-sex fights carapace and chelae length differences were less than 5 %. For mixed-sex trials animals were only matched for carapace length (within 5 % for inter-sex fights and 10 % for sexual interactions).

3.2.3 Urine visualisation procedure

Fluorescein was used to visualise urine release following the methods developed by Breithaupt & Eger (2002). A 0.3 % sodium fluorescein solution (dose 9-10 μg g^{-1} body mass) was injected into the pericardium region of crayfish three/four hours prior to experiments using a 250 μl syringe (Hamilton, Switzerland) and a 45-gauge needle (Microlance, Ireland). After injection the hole was sealed using plasticine and tape to avoid haemolymph loss and crayfish were fed on defrosted prawns. The technique was successful in visualising urine in all individuals (N = 120).

3.2.4 Social interactions

Interactions took place in a glass aquarium (40 x 20 x 20 cm) which was adapted for filming fluorescein release by covering the walls with black opaque lining. Light from a 150 W slide projector (Reflecta Diamator, AF, Germany) was reflected into the tank by a mirror (44 x 20 cm). Interactions were filmed from a front view only (Sony Hi8, CCD_VX1E).

Interactions started after a 30 min acclimatisation period, where animals were physically and chemically isolated by an opaque divider. Fights were recorded for 30 mins after the divider was lifted whilst sexual interactions were filmed until 5 min after mating ended (defined as when an animal dismounted and mating behaviour did not reoccur after 5 min). Following each experiment the equipment was washed thoroughly using carbon filtered water.

The timing of urine release and the behaviour of each crayfish was analysed in 15 male-male, 15 female-female and 15 male-female fights and 15 male-female sexual interactions.

3.2.5 Behavioural analysis

Filmed interactions were analysed using a behavioural software package (The Observer 5.0, Noldus Inc). Throughout the experimental phase both crayfish were assigned a behavioural score using a mutually exclusive scale, incorporating agonistic (adapted from Breithaupt & Eger 2002) and sexual behaviours (adapted from Stebbing 2005; Stebbing et al. 2003a) (table 3.1). A new behavioural level was identified, touching (Level 3A), which represented behaviours where animals were in contact but were non-aggressive i.e. they did not use the claws against their opponent.

Table 3.1: Definition of agonistic and sexual behaviours displayed by signal crayfish in social interactions. The behavioural level "claws not grasp" previously described by Breithaupt & Eger (2002) was split into two separate categories, level 3A touching, and level 3B claws not grasp. For full descriptions of behaviours see (Atema & Voigt 1995; Stebbing et al. 2003a).

Behaviour		**Behavioural Elements**
Agonistic behaviours	***Agonistic level***	
Fleeing	-2	Tail flipping, walking away quickly
Avoidance	-1	Walking away slowly, turning from opponent
Separate	0	Animals separate
Initiation	1	Approach or following opponent, turn towards opponent
Threat Display	2	High on legs, meral spreading
Touching	3A	Animals touching via body, antenna or chela(e) with limited movement
Physical contact (claws not grasp)	3B	Antenna whipping, claw pushing, claw boxing, claw tapping
Physical contact (claws grasp)	4	Claw lock, clamping of chela(e) onto opponents body
Unrestrained	5	Claw snapping, claw ripping
Mating behaviours		
Seizing		Male grips the female at the rostrum, chela(e) and/or antenna, usually from an angled position.
Turning		Male secures pereopods around the cephalothorax of the female (either from an adjacent position or by climbing on top of the female)
Mounting		Males holds female so ventral surfaces are facing and maintained in a parallel position.
Spermatophore Deposition		Arching and depression of the male abdomen whilst depositing spermatophores on female ventral surface. Pauses common between cycles
Dismount		Female is released from the mounting position through movement of the pereopods or chela(e).

3.2.6 Agonistic behaviours

For each interaction dominant animals were identified as those which initiated fights and showed high levels of aggression (grasping of opponents body with the claws, unrestrained aggression). Subordinate animals displayed submissive behaviours (avoidance, fleeing) and subsequently did not reengage in fights.

To identify animals displaying high levels of aggression, an aggression index was assigned to each animal by calculating the proportion of time spent displaying aggressive behaviours at levels 4 and 5 (table 3.1) in relation to all aggressive behaviours (level 3a-5).

3.2.7 Reproductive behaviour

Female crayfish were scored during sexual interactions to assess their motivation towards mating. Females were scored as i) receptive; females stretch their claws out in front of their body, which is lowered towards the substrate, aiding the male in mounting and spermatophore deposition; ii) avoidance; female flees or avoids the male (level -2,-1. table 3.1); iii) agonistic; female displays characteristic aggressive behaviours found in fighting (level 1 to 5. table 3.1), or, resists seizing, turning and mounting by the male by pushing, boxing or clamping onto the male using chelae/pereopods; iv) isolation; female in isolation prior to any social interactions.

3.2.8 Urine release analysis

The release of stained urine was recorded for both individuals during the acclimation and experimental period. A measure of urine output was determined for each individual from the time spent releasing urine (percentage of time spent releasing urine in relation to the total time animals were in contact).

3.2.9 Statistical analysis

Prior to parametric analysis data was tested for normality (Kolmogorov-Smirnov test) and homogeneity (Levene's test). If parametric test assumptions were violated equivalent non-parametric analysis was performed. All data on the percentage urine duration was arcsine square root transformed to meet test assumptions. A two-way repeated measures ANOVA was used to investigate differences in urine release between social activities (between subject factor: male fight, female fight, mixed-sex fight, sexual interaction) and crayfish dominance (within subject factor). In mixed-sex fights and sexual interactions male crayfish were identified as the dominant animal whilst female crayfish were subordinate. Planned comparisons were performed as post-hoc analysis using contrast tests (JMP IN 5.1, SAS Institiute Inc) adjusted for multiple testing using the modified Bonferroni correction (Legendre & Legendre 1998).

Comparisons of female urine release during different motivational behaviours in sexual interactions were analysed using a Friedman test. Wilcoxon signed rank comparisons were used as post-hoc tests (adjusted using the modified Bonferroni correction, Legendre & Legendre 1998).

A Pearson correlation was performed to assess if animals displaying a high aggression index also showed a high level of urine release. A one-way ANOVA was used to compare the aggression index of animals between male fights, female fights and mixed-sex fights. A Tukey's HSD test were used for post-hoc comparisons.

3.3 Results

3.3.1 Sex-specific differences in chemical signalling

Crayfish displayed sex-specific differences in urine release during agonistic and sexual interactions. Female crayfish released urine for a similar duration irrespective of whether they were engaged in same-sex fights, mixed-sex fights or sexual interactions (figure 3.1; table 3.2; two-way ANOVA; $F_{3,56} = 9.57$, $p < 0.001$; post hoc contrast tests: $p < 0.05$). However, male crayfish reduced urine signalling when engaged in sexual interactions in comparison to mixed-sex fights and male fights (post hoc contrast test; $p < 0.05$).

Table 3.2 Two-way repeated measures ANOVA results for the release of urine during different social activities. Social activity – between subjects factor, dominance – within subject factor.

Factor	df	F	p
Social activity	3	7.61	< 0.001
Dominance	1	5.65	0.02
Interaction	3	9.57	< 0.001
Total	56		

Female crayfish released urine for significantly longer than male crayfish during sexual interactions (post hoc contrast test; $p < 0.05$).

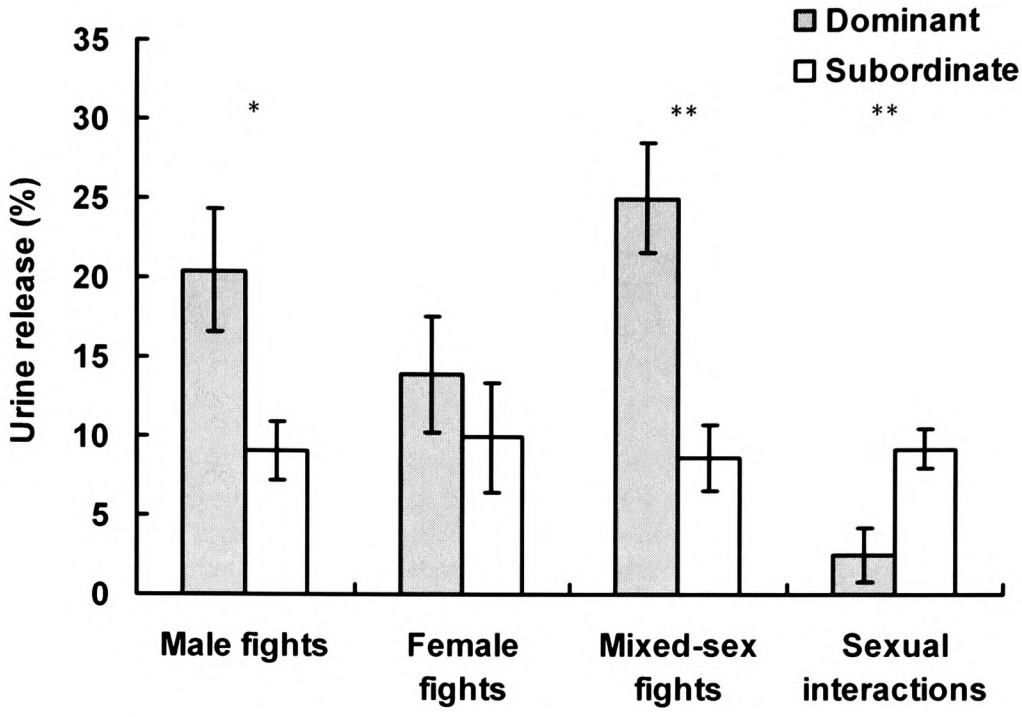

Figure 3.1: Urine release by dominant (grey bars) and subordinate (white bars) crayfish in male fights, female fights, mixed-sex fights and sexual interactions. Male crayfish were identified as dominant animals (grey bars) in mixed-sex fights and sexual interactions. Female crayfish released urine for a similar duration in all social interactions whereas males crayfish significantly reduced urinary signalling during sexual interactions in comparison to male fights, and mixed-sex fights. Values are means ± S.E.M. ** denotes significance within experiments ($p < 0.05$), * denotes a trend ($p = 0.053$).

3.3.2 Mating behaviour

Sexual interactions followed a characteristic pattern which has been previously observed in *P. leniusculus* (Stebbing et al. 2003a) taking on average 23 minutes to complete.

During sexual interactions agonistic behaviours preceded mating and could reach levels of unrestrained aggression (Level 5; table 3.1). Precopulatory fights lasted on average 226 sec (± 79.2 sec). Mating commenced when the male seized the female at the rostrum, antennae or chelae and tried to turn her so ventral surfaces were facing and spermatophores could be deposited. Females exhibited agonistic behaviours prior to and at the onset of mating but showed receptive postures before the males could successfully deposit spermatophores (figure 3.2).

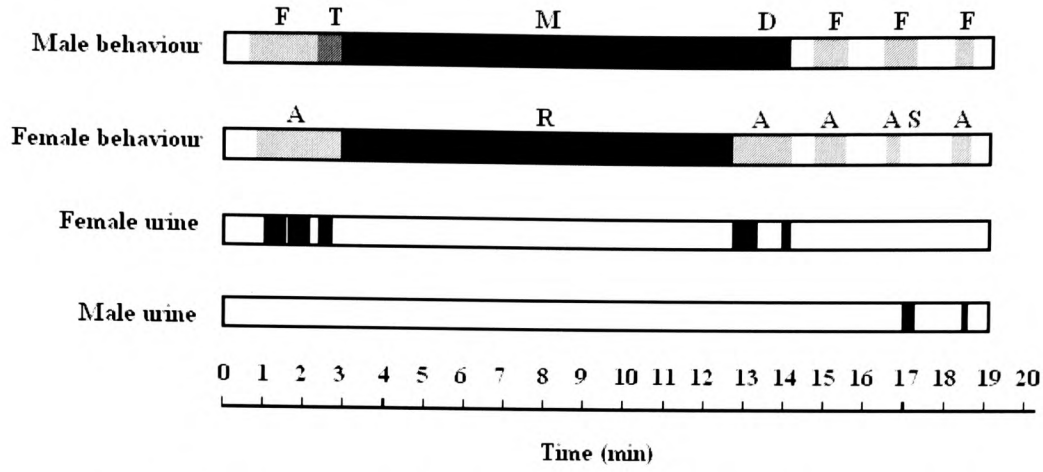

Figure 3.2: Ethogram of a an example sexual interaction between signal crayfish. Male behaviours displayed were fighting (F) (behavioural level 1 – 5, see table 1); seizing/turning (T); mounting/deposition (M) and dismounting (D). Female behaviours shown were aggressive (A); receptive (R) and submissive (S); see methods for behavioural descriptions. Female and male urine release is denoted by black bars. White sections show times when animals were separate. Urine release was associated with aggressive behaviours from female crayfish.

3.3.3 Female urine release in sexual interactions

All females released urine prior to mating, with 94 % of females releasing urine during pre-copulatory aggression. In comparison a third of males did not release any urine prior to mating. Urine release by females primarily occurred during aggressive behaviours in relation to other behaviours (figure 3.3; Friedman test; $\chi^2 = 27.14$, df = 3, $p < 0.001$). Females released urine for a longer duration whilst displaying aggressive behaviours in comparison to when females displayed receptive behaviours (Wilcoxon signed rank; $Z = -3.14$, $p < 0.01$), acted submissively (Wilcoxon signed rank; $Z = -3.30$, $p < 0.01$) or were not engaged in social interaction (Wilcoxon signed rank; $Z = -3.30$, $p < 0.01$).

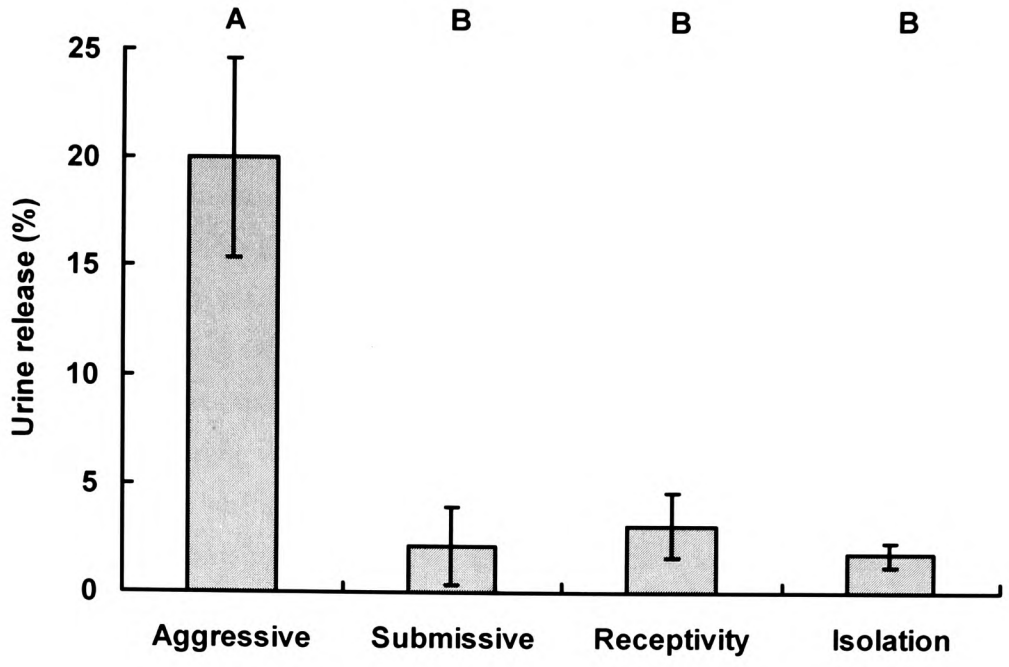

Figure 3.3: Urine release during different categories of behaviours displayed by female crayfish. Values are mean urine release as a percentage of total time spent displaying each behaviour (± S.E.M). Lettering denotes significance ($p < 0.01$).

3.3.4 Agonistic behaviour

Fights between female crayfish and inter-sex fights both followed the characteristic pattern that has been previously described for adult male crayfish (Goessmann et al. 2000; Breithaupt & Eger 2002). The winner of a fight displayed more aggressive behaviours (table 3.1; level 3b - 5) in comparison to the subordinate animal which did not re-engage in fight bouts following displays of submissive behaviours (levels -1, -2).

There was a significant difference in the proportion of time crayfish spent displaying highly aggressive behaviours (high aggression index) between different fights (one-way ANOVA; $F_{2,87} = 0.67$, $p < 0.001$). Male fights were more aggressive than female (Tukey's HSD test; $p < 0.001$) and mixed-sex fights (Tukey's HSD test; $p < 0.001$), whereas similar levels of aggression were shown in female fights and mixed-sex fights.

3.3.5 Urine release in agonistic interactions

During fights urine release was associated with aggressive behaviours: individuals with a high aggression index released urine for longer durations than crayfish with low aggression indices (Pearson Correlation; $r = 0.28$, $p < 0.001$, $N = 90$).

3.4 Discussion

Previous studies have suggested that urinary cues play a key role in coordinating the mating behaviour of decapod crustaceans (Atema & Steinbach 2007). Using visualisation techniques during social interactions, that urine release in male and female crayfish (*P. leniusculus*) coincides with aggressive behaviours rather than reproductive behaviours. Both sexes release urinary signals in same-sex and mixed-sex fights. During the breeding season, females interacting with males release urinary pulses when showing strong resistance prior to the onset of mating. Males reduce or discontinue urine release during sexual interactions, as compared to agonistic encounters. The results presented here suggest that the release of urine in social interactions represents an adaptation to sending an aggressive signal rather than a courtship signal.

3.4.1 The role of female urinary cues in eliciting male courtship

Previous findings have shown that male crayfish respond behaviourally and physiologically to stimulation with exogenous female odour (urine and conditioned water), indicating sex recognition through female urinary cues (Stebbing et al. 2003a; Stebbing et al. 2004; Berry & Breithaupt 2008). In contrast, the timing of urine release suggests that urine is generally used as an aggressive signal by females, rather than as a courtship stimulating signal. Male courtship behaviour could be triggered by other non-urinary female cues. However, behavioural experiments on crayfish, lobsters, and different species of crabs indicate that male courtship is generally elicited by female urinary signals (Hardege et al. 2002; Stebbing et al. 2003a; Atema & Steinbach 2007). Furthermore in our study, female urine delivery was observed prior to any seizing attempts by male crayfish. This provides an opportunity for males to receive information about sex and receptivity and can subsequently elicit male mating behaviour.

Like females, male urine release coincided with aggressive behaviours, which were displayed during fights. Males did not release urine during reproductive behaviours. During breeding season, on recognition of a receptive female, male crayfish altered their posture, reduced the level of aggressive signalling towards the female and switched to mating behaviour.

3.4.2 Absence of male urine release during courtship

Female crayfish invest substantial resources into rearing their offspring, it is therefore expected that they actively choose dominant male partners. Dominant males should therefore actively advertise their status during courtship (Andersson 1994). Components of the male dominance signal might be comparable to components of aggressive signals released during male fights, as has been found in the cockroach mating system (Moore & Moore 1999; Moore et al. 2001). However, our results appear to contradict this possibility, with male crayfish reducing signalling as a result of recognising a potential female partner. Thirty percent of the crayfish males did not release any urine during sexual interactions. Rather than solely relying on chemical signals to choose their mate, female crayfish may exhibit multi-modal choices based on chemical, visual and/or mechanical traits (Acquistapace et al. 2002).

3.4.3 Sexual conflict and urine signalling

For most animal species there is unequal investment in gametes and progeny between the sexes, with males investing less than females (Trivers 1972). Males consequently have a higher potential reproductive rate and compete over females, whilst females are more resistant and are only interested in mating with the best available male (Clutton-Brock & Parker 1992). Strong asymmetries between the evolutionary interests of the sexes inevitably results in sexual conflicts (Jormalainen 1998).

Sexual conflict is expected to be particularly influential on mating strategies of crayfish due to the considerable difference in parental investment between the sexes (Gherardi 2002). The results presented in this study support this hypothesis as receptive females do not actively advertise their receptivity with urine signals. Instead, during pre-copulatory aggression, females release urine in an attempt to repel the male rather than as an invitation to mate.

Urine release during fighting behaviours follows the same pattern in male and in female crayfish, suggesting a function as an aggressive signal in both sexes. However, since urine contains hormonal metabolites, its release can convey additional (potentially unwanted) information about female receptivity, which can be exploited by the male to identify a mate. Females, therefore, appear to miscommunicate their aggressive motivation by eliciting courtship rather than a defensive behaviour in the male.

In dense populations, female crayfish may actually avoid dominant males in order to reduce the likelihood of suffering injuries from aggressive interactions. In light of the aggressive function of urine signalling, the reduction of male urine release during courtship can be regarded as an appeasement towards the female. It may serve the male to de-escalate female aggression and to increase female motivation to mate.

3.4.4 Urinary signalling in agonistic interactions

Urine release during agonistic behaviours follows the same pattern in male and in female crayfish, suggesting a function as an aggressive signal in both sexes. The level of aggressive signalling found in this study supports previous work showing urinary threat signals influence the outcome of fights; potentially by revealing information about the motivation and physiological state of the sender (Breithaupt & Eger 2002). This study has shown crayfish release urine under specific behavioural conditions, primarily when engaged in physical, agonistic contact. Releasing urine when opponents are in close proximity increases the signal to noise ratio, and can enhance the efficacy of the signal.

Female fights were found to be less aggressive than males, with lower overall levels of urine release and dominant animals releasing similar amounts as subordinates. A reduction in the time spent displaying aggressive behaviours by female crayfish implies they are less competitive than male crayfish. Sexual selection theory predicts males will be inherently more competitive than females (Darwin 1871) as males need to compete for access to females, whereas females invest more energy and resources into their offspring and less into pair formation. Large male crayfish have been shown to have a clear advantage over small subordinate crayfish, having increased access to food and mating opportunities (Villanelli & Gherardi 1998; Gherardi et al. 2006). Females do not appear to be able to compete with dominant males and instead act like subordinate males during dominance hierarchy formation, potentially to avoid injury.

3.4.5 The 'dilemma' of urine signalling

The results presented here add to the growing body of research which show that decapod crustaceans use urine borne signals to mediate a variety of intraspecific interactions (Atema & Steinbach 2007). As a source of pheromone communication urine can carry multiple messages, which can be uncheatable information about the physiological state and identity of the sender. Receivers must be able to decipher active pheromone signals amongst a complex mixture of general metabolic products.

Our results show that information about the sex and status of the female signaller is conveyed to the male receiver in pre-copulatory aggression. Unlike moths (Greenfield 1981; Alexander et al. 1997), females do not appear to be actively signalling their receptivity to males through a long distance pheromone. Alternatively, females released urine as an aggressive threat signal that males were able to exploit by detecting urinary metabolites, which signalled the female's sex and receptive state. 'Spying' of hormones and metabolites in female urine has been found in the sex-recognition system of multiple fish species (Sorensen & Stacey 2004; Stacey & Sorensen 2005). To understand if 'spying' is adaptive for female crayfish further research into the chemical identity of the active pheromone components of the urine and their behavioural significance is required. This would help to confirm that female urinary signals contain information of their sex and receptive state and aid understanding of how social behaviours in crayfish are mediated.

3.4.5 Conclusions

Chemical communication plays an important role in the mate choice of many arthropod species, with females advertising their status and sex to male receivers. Female insects may utilise pheromonal signalling as a way to avoid high predation risks, however for decapod crustaceans the reasoning behind female signalling is not as clear. Male *P. leniusculus* appear to have evolved a spying mechanism to recognise the sex and status of female crayfish from urinary signals which have evolved to act as threat signals. It remains unclear if this system is present for all decapod crustaceans.

Chapter 4

Mate choice in crayfish, *Pacifastacus leniusculus:* Do females choose dominant partners when given a free choice?

4.1 Introduction

In most animal species, there are asymmetries in the cost of gamete production and parental care between the sexes, with females having a higher investment in their offspring than male partners (Andersson 1994). Females are therefore the 'choosier' sex and in order to maximise their reproductive success individual's try to mate with the 'fittest' male available (Trivers 1972). It is therefore expected that females choose dominant males (winners of male-male combats) to gain direct benefits, such as access to limiting resources, or, indirect genetic traits which result in increased fitness of their offspring. Males, however, can increase their reproductive success by maximising their number of mating partners, which is usually achieved by out-competing other males for access to mates.

Secondary sexual characters in many species can function both in male-male competition and as cues for female choice (Andersson 1994). However the effects of female mate choice can be extremely difficult to disentangle from male-male competition, which can result in dominant males forcefully excluding subordinates from potential partners and therefore preventing a free female choice. The removal of intra-sexual competition, by tethering male opponents, has shown that dominant males are not always preferred by females (Östlund-Nilsson & Nilsson 2000; Zhang et al. 2006). In some cases the cost of choosing the dominant male might actually outweigh the benefits of mating; through increased risk of injury or death, increased disease transmission or a reduction in fertilisation due to sperm depletion (Qvarnström & Forsgren 1998).

In many crustacean mating systems there is morphological asymmetry between the sexes, with males being of larger size and having enhanced weaponry (claws) in comparison to females. Males therefore have the potential to overpower their partners and be sexually coercive (Clutton-Brock & Parker 1995). Despite this, previous results have indicated that females can actively choose partners, and resist mating with unsuitable males (Snedden 1990; Jormalainen 1998; Sutherland et al. 2007).

Female crayfish have been shown to preferentially choose large males, with large, symmetrical chelae (Villanelli & Gherardi 1998; Gherardi et al. 2006; Aquiloni & Gherardi 2008c). Large body size and chelae are related to the ability to win agonistic encounters and therefore gain access to and defend limiting resources, such as shelters

(Bergman & Moore 2003). It is particularly important for female crayfish to select a good quality mate as they invest substantial energy reserves and time into rearing their offspring. Female signal crayfish spawn in the autumn following copulation, with the eggs developing under the abdomen and are guarded through winter until they hatch the following spring. During this time females minimise feeding and continuously fan and groom the eggs, restricting predator escape responses (Reynolds 2002).

Previous work has speculated that female crayfish might be able to distinguish quality partners by chemical cues (Aquiloni & Gherardi 2008a), these cues could potentially be released in the urine (Bushmann & Atema 2000). However, y-maze choice test trials comparing female choice of odours from dominant and subordinate crayfish have remained inconclusive (Zulandt Schneider et al. 1999). Female crayfish only showed a trend for spending more time in the dominant male compartment in comparison to the subordinate. This may be a result of the relatively small sample size used in the experiment (N = 7) and animals were not tested for receptivity prior to trials.

Studies of the mating system of cockroaches have indicated that females choose dominant males using chemicals which are comparable to components of threat signals released during male-male competition (Moore 1997; Moore & Moore 1999; Moore et al. 2001). Manipulation of the chemical signature of males can have an effect on the choice of the female cockroach, with females altering fecundity in relation to changes in odour cues (Moore et al. 2001). Using similar mechanisms it could be hypothesised that female crayfish could potentially recognise components of the male dominance signal, which are released during agonistic interactions (chapter three; Breithaupt & Eger 2002). Recent studies have contradicted this theory, showing that females were unable to choose dominant males over equally sized subordinates (Aquiloni et al. 2008; Aquiloni & Gherardi 2008c). However these experiments did not study any mating behaviours (seizing, turning, mounting, spermatophore transfer) and females were only given the chance to choose their mate over a relatively short time scale, 20 min, 30 min (Aquiloni et al. 2008; Aquiloni & Gherardi 2008c). Previous studies (chapter three) have indicated that signal crayfish can take over 30 min to complete mating, therefore in this experiment animals will be allowed to interact for longer time periods (overnight). This should allow the full suite of mating behaviours to be observed and the number of mating opportunities for each female will be maximised.

This experiment aims to investigate if female crayfish (*Pacifastacus leniusculus*) will preferentially mate with dominant male crayfish when given a free choice. Size-matched male crayfish, of differing social status, will be tethered to prevent male competition but allow mating. If females can distinguish dominance cues it would be expected that she would preferentially mate with the male of higher status.

4.2 Materials and methods

4.2.1 Animal maintenance

Adult signal crayfish (*P. leniusculus*) were obtained in August 2007 from a crayfish dealer (Chris Campbell, North Dorset) having been trapped in a local lake (temperature 18°C). This ensured animals were caught prior to the start of the breeding season. Animals were separated by sex and housed in a recirculating freshwater system containing six tanks (45 x 60 x 30cm) with a carbon filter (Pozzani Pure Water PLC, UK) and a UV filter (Lotus Water Garden Products, UK). A maximum of 25 crayfish were kept in each tank. Temperature and light conditions were gradually reduced to 10°C, 10:14 h light: dark cycle, which reflected natural conditions for the breeding season. Crayfish were fed twice weekly, including on the morning of the experiment, with commercially available, defrosted prawns.

To study female mate choice 22 adult intermoult female crayfish (mean ± SE carapace size of 35.4 ± 0.7 mm, mass 33 ± 1.9 g) and 44 male intermoult crayfish (mean ± SE carapace size of 38.3 ± 0.5 mm, mass 40.2 ± 1.4 g) were used, all with intact appendages. Prior to agonistic interactions male crayfish were size matched, within 5 % for post-orbital carapace length and weight, and within 6 % for cheliped length (average of the two claws).

Animals were checked for receptivity prior to mate choice experiments. Following an acclimatisation period of 10 min, behaviour of a male and female adult crayfish was observed for 20 min in a glass aquarium (30 x 20 x 20 cm). Males were classed as sexually receptive if they tried to turn or mount the female. Females were receptive when they showed a 'receptive posture'; in this position the claws are outstretched in front of their body, which is lowered towards the substrate, aiding the male in mounting. In addition females could be identified as sexually receptive if glair glands were visible as whitened tissue on the underside of the telson. Unreceptive animals were returned to holding tanks.

Experiments were carried out during the breeding season, Oct – Nov 2007. To reduce any influence of previous social interactions, one week prior to experimentation, crayfish were isolated in 3 l containers (24 x 17.5 x 8 cm). Each individual was numbered using white corrective fluid as a visual marker on the carapace to aid identification during analysis.

4.2.2 Fight interactions

Prior to mate choice experiments the social status of size-matched male crayfish was determined through paired contests. Fights took place in a glass aquarium (40 x 20 x 20 cm) which had three of the side walls covered with black opaque lining to prevent visual disturbances. The aquarium was lit from above by a 60 W lamp. After a 15 min acclimation period animals were observed until five min after dominance status had been established. Subordinate animals were defined as those which showed submissive behaviours (avoidance, fleeing) and subsequently did not display highly aggressive behaviours (grasping of opponents body with the claws, unrestrained aggression). The dominant male was the winner of the fight and continued to show high levels of aggression (claw grasping, pushing) once status was established.

4.2.3 Mate choice experiments

Mate choice experiments took place in a glass tank (90 x 30 x 30 cm), filled with carbon filtered water. The tank was covered with black opaque lining to prevent visual disturbances and reflections. Opaque screens were used to divide the tank into three equal compartments. Mate choice experiments were designed so females could have free access to two male crayfish, whilst preventing male-male competition (figure 4.1). Males of differing dominance status (as determined by fight interactions) were tethered to each end of the tank (randomly assigned) using thin nylon thread. Tethering allowed males to display normal postures and mating behaviours but confined their movement within their individual compartment. A female crayfish was placed in the middle section of the tank. After an acclimation period of 20 min, a removable 10 cm section was lifted from the opaque screen which allowed the female to have free access to males but prevented males from visually seeing each other.

Experiments were filmed using a time lapse recorder (Sony; SVT-124P) and a wide angle camera mounted 1 m above the tank. The recorder captured data (12.5 images per

sec) on the behaviour of each animal over a 16 hour period (approximately 5pm until 9am). The tank was lit using a 40 W red light.

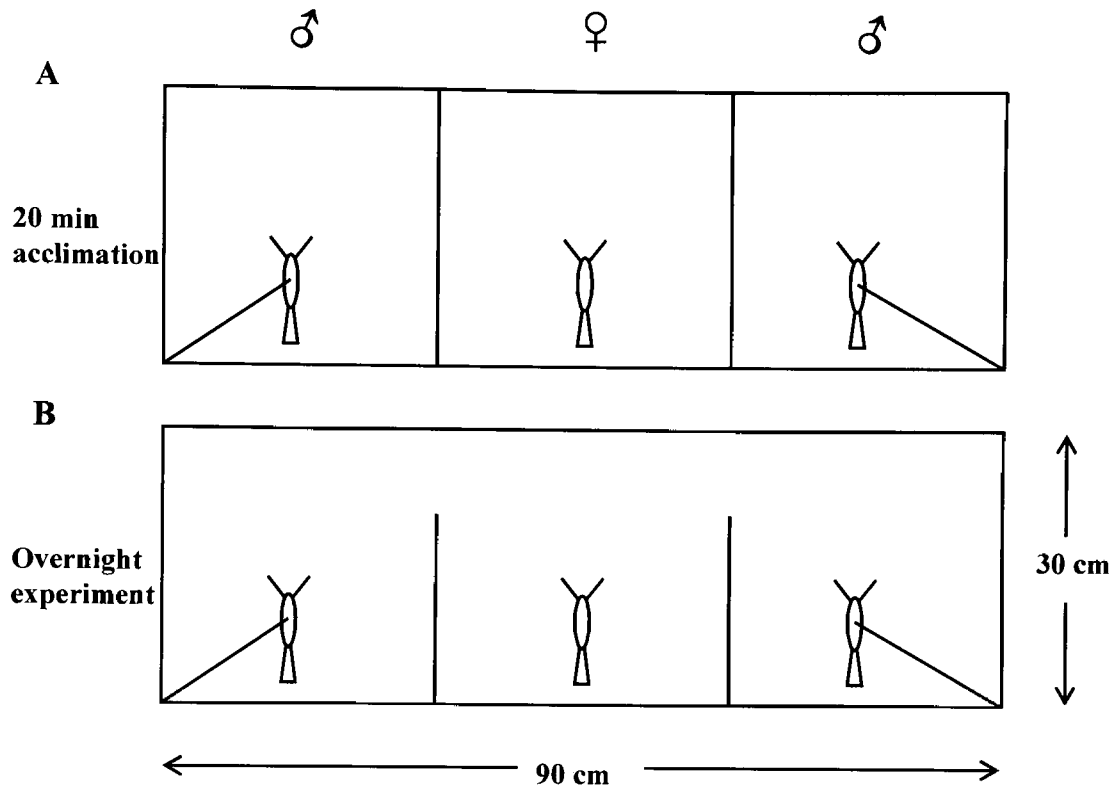

Figure 4.1: Experimental set-up of mate choice experiments (as viewed from above) (A) Set-up during 20 min acclimation period where opaque dividers physically and chemically separated two tethered male crayfish from a female crayfish; (B) Experimental set-up, after a 10 cm section of each divider was removed. Tethering allowed males to display normal postures and mating behaviours but confined their movement within their individual compartment.

4.2.4 Mate choice analysis

Female preference for male dominance was tested using four criteria; (1) It was assessed if females preferentially mated with dominant males in comparison to subordinate males. Mating was defined as occasions where the male turned the female and deposited spermatophores on the ventral surface. Occasions where males attempted to seize and turn the female but were unable to successfully mount and deposit spermatophores were recorded and used to estimate the mating success rate for dominant and subordinate males. The number of matings and the duration of mating events were recorded over the full duration of the experiment. (2) Female choice was assessed by recording the status of the male crayfish which the female visited first. (3) To determine if females preferred dominant males we recorded the time spent in each

male compartment for the first hour of the experiment (when females were most active). (4) Aggressive interactions are usually a precursor to mating, therefore the duration of agonistic bouts (where individuals used the chelipeds in aggression) was also recorded for the first hour of the experiment.

4.2.5 Statistical analysis

Female choice of dominant and subordinate males (first choice, mate choice) was compared using a binomial test. Data on the time that female crayfish spent in each male compartment and the time spent engaged in agonistic interactions was tested for normality (Kolmogorov-Smirnov test) and comparisons between dominant and subordinate males were made using a paired t-test. For this analysis, only experiments where the female visited both compartments within the first hour were included (N = 20).

4.3 Results

Females displayed no preference towards size-matched male crayfish of differing status (table 4.1). Females did not preferentially visit dominant males before subordinate males (11 first visits to dominant, 11 first visits to subordinate) and in the first hour of the experiment females did not spend significantly more time in the dominant male compartment compared to the subordinate (Paired t test; $p = 0.77$). In addition females did not engage in agonistic interactions for significantly longer with dominant males in comparison to subordinate males (Paired t test; $p = 0.24$).

Female crayfish did not preferentially mate with dominant males in comparison to subordinate males. Eight matings were recorded where spermatophores were successfully transferred to the female; 4 matings with subordinate males and 4 matings with dominant males. In one experiment the female mated with both males. The female mated with the subordinate male first, then with the dominant. However, mating was longer with the dominant male (1 hr 20 min), in comparison to the subordinate (23 min).

Dominant males were more successful than subordinate males at mounting females and transferring spermatophores; once they had seized the female 80% of mating attempts were successful for dominant males, in comparison to 60% for subordinate males. In one experiment the same male could mate with the female multiple times, with one dominant male mating with the same female five times. Dominant males spent a total of

5 hours mating with female crayfish, in comparison to only 2 hrs 35 min for subordinate males (comparison of four dominant and four subordinate males which transferred spermatophores in the experiments).

Table 4.1: Mate choice and behaviour among female crayfish (*P. leniusculus*) interacting with two tethered male crayfish. Male crayfish were size-matched but had differing social status. In the first hour of the experiment females did not spend significantly longer in the dominant male compartment or engaged in aggressive interaction with the dominant male ($p > 0.05$; Paired t test).

Behaviour	**Dominant male**	**Subordinate male**	**Significance**
First choice	11	11	$p = 1$ (Binomial test)
Successful matings	4	4	$p = 1$ (Binomial test)
Time spent in compartment (min)	25.47 ± 03.26	23.42 ± 03.34	$p = 0.765$ (Paired t test)
Aggressive contact (min)	3.12 ± 00.50	2.10 ± 00.41	$p = 0.236$ (Paired t test)
No of mating attempts /successful mating	10 / 8	10 / 6	

4.4 Discussion

Female selection of dominant male partners is believed to be the major driving force behind secondary sexual traits and sexual selection (Andersson 1994). However, female crayfish (*P. leniusculus*) were found to show no preference for dominant male crayfish. When given a free choice, females did not preferentially visit, or, spend more time engaged in mating or agonistic interactions, with dominant males in comparison to size-matched subordinate males.

The results presented here support previous studies which have shown that females can not distinguish dominant males when exposed to male odours (Aquiloni & Gherardi 2008c), or, when given a free choice over a short time scale (Aquiloni et al. 2008). Female crayfish have been shown to choose large partners, which can mate with females more frequently and for longer than subordinate males (Villanelli & Gherardi 1998; Gherardi et al. 2006). These results imply that while females can distinguish

distinct morphological traits (such as increased size) they can not distinguish equally sized males based on chemical cues advertising status.

Recent trials using fluorescein as a visual marker for urine have shown that male crayfish reduce urinary signalling during courtship as a result of recognising a potential female partner (see chapter three). During agonistic interactions urinary pheromones are released as an aggressive signal by both sexes (chapter three; Breithaupt & Eger 2002), therefore males may reduce signalling during pre-copulatory aggression as an indication of their willingness to mate. However this raises questions as to how females determine the quality of their mate and what signals males use to advertise their status. Previous studies have indicated that females may require multi-modal signals to choose their mate potentially based on chemical, visual and/or mechanical male traits (Aquiloni & Gherardi 2008a). Aquiloni et al. (2008) have proposed that females are more likely to distinguish dominant partners after eavesdropping male-male combats, suggesting females are able to indirectly obtain information on male status. During these trials female crayfish could see and smell cues from fighting males, so it is unsure whether visual or chemical cues were of higher importance in distinguishing male status.

In exhibiting pre-mate choice, females may delay or resist mating in order to gain an increment to their fitness, potentially by mating with a superior male or in more favourable ecological conditions. Our experiments supports this theory as subordinate male crayfish were less successful at depositing spermatophores on the female after initiating mating than dominant males (60 % success subordinate males in comparison to 80 % success for dominant males).

Sexual coercion, where males mate with females through physical force, can occur in crayfish mating systems where males are larger in size and more aggressive than females. Therefore, rather than displaying a pre-mate choice of male partners, female crayfish may exhibit a cryptic post-copulatory mate choice. This could be achieved by physically removing unwanted spermatophores (pers. obs) or by adjusting reproductive effort in relation to male traits (Galeotti et al. 2006). Previous studies have found that female crayfish (*Austropotamobius italicus*) can adjust the size of their eggs and the clutch size in relation to male traits such as body size and claw size (Galeotti et al. 2006). The size and weight of the egg clutch and of juveniles is larger when sired by

larger fathers, which the females actively choose as partners (Aquiloni & Gherardi 2008b).

Signal crayfish can live in high density populations which can result in multiple males having the opportunity to mate with the same female. In this experiment both subordinate and dominant opponents were found to mate with the same female. Therefore future experiments on female mate choice should focus on performing paternity analysis of offspring to determine if females preferentially fertilise their eggs with spermatophores of dominant males. Previous studies, which manipulated male partners of female crayfish (*Orconectes rusticus*), have indicated that the last male to mate has a paternity advantage over previous partners (Snedden 1990).

Chapter 5

Ventilatory and heartbeat activity of crayfish, *Pacifastacus leniusculus*, during reproductive behaviours

5.1 Introduction

Measuring the physiological state of aquatic crustaceans has provided insights into internal responses to fluctuating environmental conditions (McMahon 1999; McMahon 2002). Crustaceans have been found to alter cardiac/ventilatory activity in response to changes in salinity/oxygen (McGaw & McMahon 2003), temperature (Ahsanullah & Newell 1971; DeWatcher & McMahon 1996), light (Cumberlidge & Uglow 1977; Li et al. 2000), toxic substances (Bini & Chelazzi 2006) and following controlled exposure to biologically important chemicals, such as adenosine (Stegen & Grieshaber 2001) and serotonin (Listerman et al. 2000). Physiological responses can be extremely sensitive indicators of stimulus perception when no external behaviours are displayed (Schapker et al. 2002).

The development of non-invasive, infrared, methodologies has allowed physiological parameters to be measured in combination with behavioural analysis (Depledge & Andersen 1990). To date studies on aquatic crustaceans have focused on measuring cardiac activity and ventilatory activity during agonistic interactions (Schapker et al. 2002) or locomotion (McMahon 2002). For example, ventilation and heart rate have been found to increase during aggressive behaviours (Schapker et al. 2002).

There is currently heightened interest in studying the reproductive behaviours of invasive crayfish species. In an effort to control nuisance populations pheromone baits have been proposed as a way of increasing trap efficiency (Stebbing et al. 2003b; Stebbing et al. 2004). Prior to employing pheromone baits it is important to know the active chemical component of crayfish sex pheromones. Recent attempts to design a novel bioassay to aid the identification of crayfish sex pheromones have been unsuccessful due to contradictory results and reliance on non-sexual behaviours as indicators of positive responses (Stebbing et al. 2003a; Belanger & Moore 2006). The development of physiological assays may provide a quick and reliable assay which can be coupled with analytical chemistry techniques to chemically identify crayfish sex pheromones. However, to date, there has been limited research on the physiological response of crustaceans to sex pheromones (Bublitz 2007).

Diversification of bioassay techniques in insect and fish systems has led to an increased use of physiological parameters to aid pheromone identification (Chapman 2000; Stacey

& Sorensen 2005). Measurement of an individual's physiological response to a specific stimulus can help provide a quick and reliable assessment of receptivity. Electro-olfactograms have helped to identify pheromones for a number of fish species including the sex pheromone of the invasive sea lamprey, *Petromyson marinus* (Li et al. 2002). Increases in the ventilation rate of the round goby, *Neogobius melanostomus*, have been used as an indicator of receptivity of steroids which may be used as sex pheromones (Murphy et al. 2001; Belanger et al. 2006). Physiological assays therefore may provide an effective way to study the responses of male crayfish to female sex pheromones.

Before physiological assays can be used in bioassay driven purification techniques it is important to ensure measures are ecologically relevant and represent a natural and regular element of the receiver's response. Therefore prior to assay development physiological parameters (heart rate, ventilation rate) need to be recorded during reproductive behaviours to assess if consistent and reliable changes occur.

This project aims to assess changes in the ventilatory activity of male and female crayfish, and the cardiac activity of female crayfish, during reproductive behaviours. This should help to indicate whether ventilation rate and/or heart rate would be biologically relevant parameters for use in a novel assay design for identification of crayfish sex pheromones. It is expected that physiological parameters will increase during reproductive behaviours in a similar way to observed increases during agonistic behaviours (Schapker et al. 2002). This increase may be more pronounced in female crayfish as they have been found to release more urinary signals than male crayfish during reproduction (see chapter 3), which is associated with elevated levels of ventilatory activity (see chapter 6).

5.2 Materials and methods

5.2.1 Animal holding conditions

Adult signal crayfish (*Pacifastacus leniusculus*) were obtained in Aug 2007 from a crayfish dealer (Chris Campbell, North Dorset) having been trapped in a local lake (temperature 18°C). Animals were separated by sex and housed in a recirculating freshwater system containing six tanks (45 x 60 x 30cm) with a carbon filter (Pozzani Pure Water PLC, UK) and a UV filter (Lotus Water Garden Products, UK). A maximum of 25 crayfish were kept in each tank. Temperature and light conditions were

gradually reduced to 10°C, 10:14 h light: dark cycle, which reflected natural conditions for the breeding season.

Measurement of physiological parameters was only successful in 15% of attempted trials, due to animals failing to display reproductive behaviours (15 trials) or due to signal failure for at least one sensor, which resulted in trials being stopped prematurely (7 trials). Physiological parameters were successfully recorded in four trials; therefore only preliminary results could be gathered.

To study ventilation rates during mating encounters we used four intermoult male signal crayfish (mean ± SE carapace size of 33.0 ± 2.1 mm, mass 30.6 ± 7.2 g) and four intermoult female signal crayfish (mean ± SE carapace size of 39.5 ± 1.3 mm, mass 38.5 ± 5.1 g), all with intact appendages.

5.2.2 Mating interactions

The ventilation rate of mating crayfish was recorded during November 2007, within the breeding season. Mating interactions took place in a glass aquarium (40 x 20 x 20 cm) and were filmed from a front view. After a 10 min acclimation period, animals were observed until 5 min after mating ended (defined as when an animal dismounted and mating behaviour did not reoccur after 5 min).

5.2.3 Physiological recordings

The ventilation rates of opponent crayfish were monitored using a non-invasive optoelectronic technique (Depledge & Andersen, 1990). One hour prior to the start of the fight an infrared sensor (SG-2BC, optosensor IR, Farnell, USA) was attached to the carapace over the right anterior branchial region of the crayfish using cyanoacrylate glue. For female crayfish only, in addition to monitoring ventilation rate, cardiac activity was measured during mating interactions. Recordings were only taken for female crayfish as attaching a heart rate sensor to male crayfish was found to hinder reproductive behaviours. One hour prior to the start of the interaction an infrared sensor (A312-95, optosensor IR, Farnell, USA) was attached to the carapace over the cardiac region of the crayfish using cyanoacrylate glue. Both sensors were comprised of a light emitting diode (950 nm) and a phototransistor detector. The phototransistor detected changes in the infrared light intensity scattered as a result of the scaphognathite movements, or, heartbeats. Signals were filtered and amplified using a two channel

amplifier (Dept. of Engineering, University of Hull) and transferred to a computer using a PowerLab AD converter (type ML880, ADInstruments). Scaphognathite movements and heartbeats were measured in mV and analysed using Chart 5.5 software (ADInstruments), which calculated an instantaneous ventilation rate and heart rate (BPM, beats per min).

Ventilation rate and heart rate was recorded for the 10 min acclimation period and the duration of the mating interaction. If an unusual behaviour was performed or a disturbance occurred in the room it was noted on the Chart file and the section of influenced ventilation rate was not analysed.

5.2.4 Physiological analysis

A mean ventilation rate (BPM) and heart rate (for female crayfish, BPM) was calculated for the different behavioural categories displayed over mating interactions. Behavioural categories were defined as (i) Isolation; the one min section prior to when interactions began, when animals were in isolation; (ii) Pre-copulatory fights; animals use their chelae to push, box or grasp their opponent prior to mating (iv) Seizing; the male crayfish grasps the female by the rostrum or antennae and tries to turn her over so that the dorsal carapace is on the ground and ventral surfaces are facing (v) Mating; the male crayfish repositions the female to facilitate spermatophore deposition on the ventral surface (vi) Dismount; the male releases the female.

5.2.5 Statistical analysis

Prior to parametric analysis all data was tested for normality (Kolmogorov-Smirnov test). A two-way repeated measures ANOVA was used to analyse if there was a significant difference in the ventilation rate of female and male crayfish during different mating behaviours (within subject factor – behaviour; between subjects factor – sex). A separate one-way repeated measure ANOVA was used to assess the heart rate of female crayfish changed during mating interactions. Where a significant difference was found post hoc analysis was performed using a Tukey's HSD test.

5.3 Results

The ventilation rate of signal crayfish changed during mating interactions depending which behaviour they were engaged in (figure 5.1; table 5.1; two-way repeated measures ANOVAs; $F_{4,24} = 6.06$, $p < 0.01$). Ventilatory activity was significantly

reduced whilst the male crayfish seized and turned the female in comparison to ventilation rate during pre-copulatory aggression and when the male dismounted the female. There was no significant difference between the ventilation rate of male and female crayfish throughout mating interactions (two-way repeated measures ANOVA; $p = 0.71$).

Table 5.1: Two-way repeated measures ANOVA results for differences in ventilation rate of male and female crayfish during reproductive behaviours. Between subjects factor- sex; within subject factor – behaviour.

Factor	df	F	p
Behaviour	4	6.06	< 0.01
Sex	1	0.15	0.71
Interaction	4	0.17	0.95
Total	24		

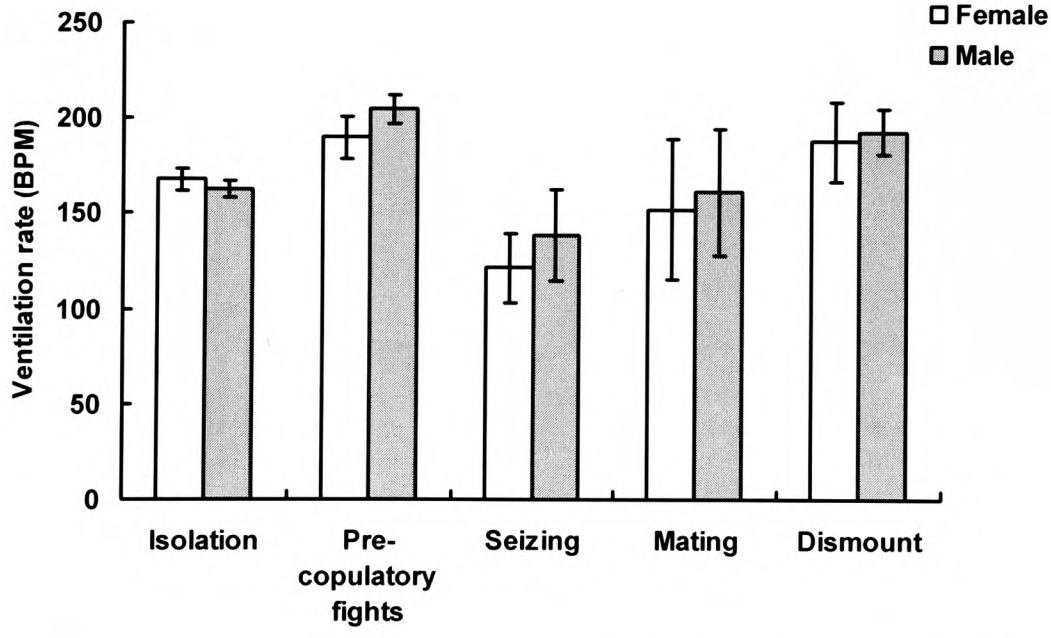

Figure 5.1: The mean ventilation rate of male and female crayfish during reproductive interactions (N = 4). Ventilation rate was significantly reduced whilst the male seized and turned the female in comparison to levels during pre-copulatory aggression and dismount behaviours (two way repeated measures ANOVA; $p < 0.01$). No difference was found between the ventilation rate of male and female crayfish.

During mating interactions, both male and female crayfish displayed ventilatory arrest, especially at the onset of seizing and turning behaviours (figure 5.2).

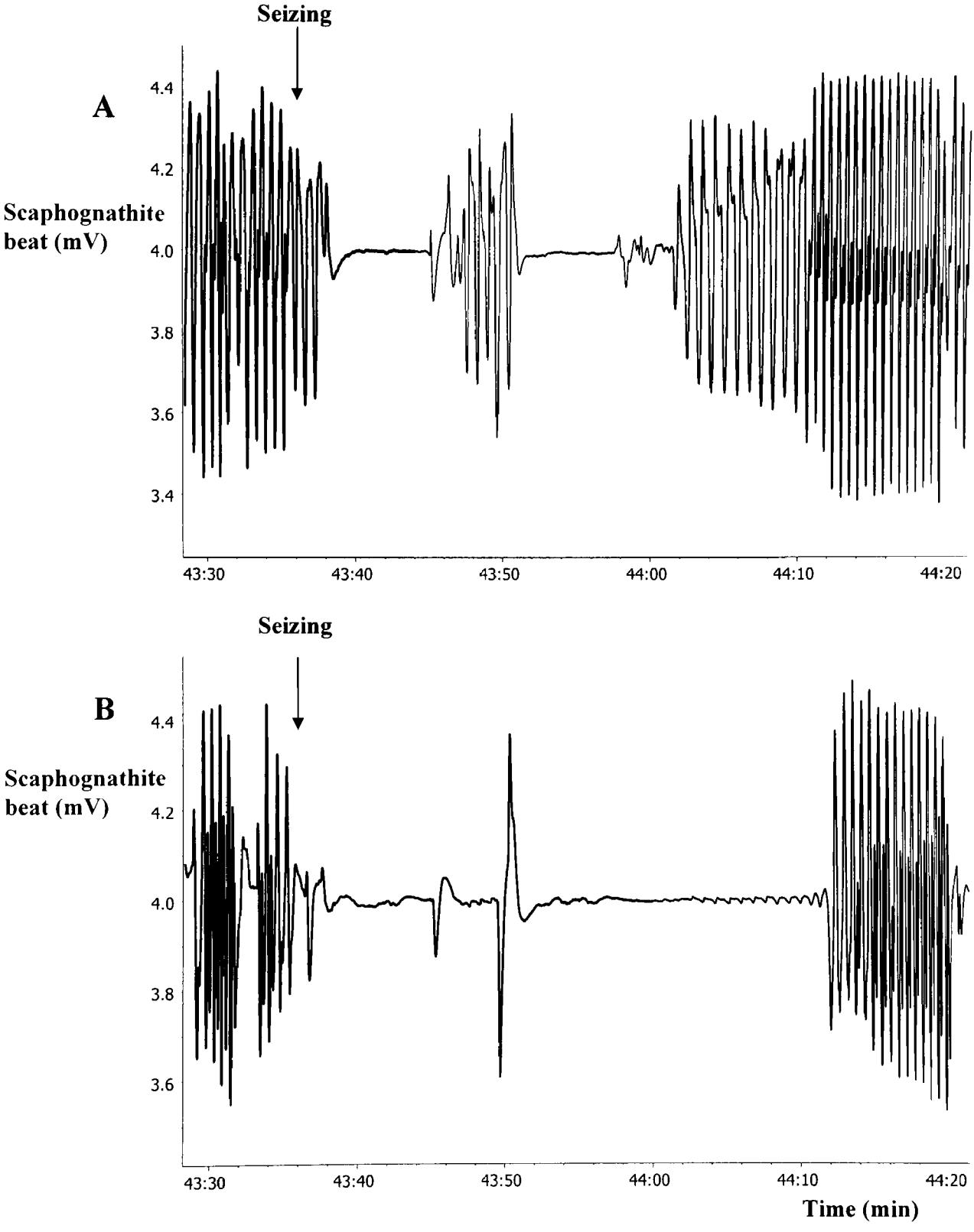

Figure 5.2: An example of the scaphognathite beat activity of (A) female and (B) male signal crayfish during reproductive behaviours. At the onset of the male seizing and attempting to turn the female both sexes showed a ventilatory arrest.

The heart rate of female crayfish showed a trend for increasing as mating progressed, however high individual variation (N = 4) appeared to prevent this trend from being significant (figure 5.3). The heart rate was significantly increased during dismount

behaviours in comparison to when animals were in isolation (One-way repeated measures ANOVA; $F_{4,12} = 4.91$, $p < 0.05$).

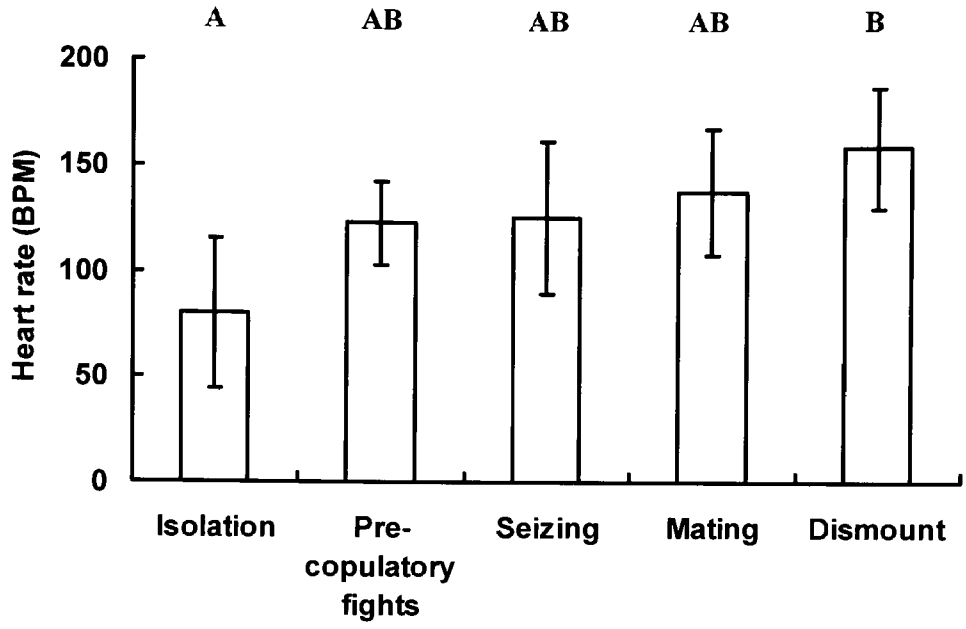

Figure 5.3: The mean heart rate of female crayfish during mating interactions (N = 4). The heart rate of female crayfish increased as a result of engaging in mating behaviours, with heart rate during dismount behaviours being significantly higher than that recorded when animals were in isolation. Bars not connected by the same letter are significantly different; $p < 0.05$, Tukey's HSD post-hoc test.

5.4 Discussion

Male and female crayfish, *P. leniusculus*, showed distinct physiological responses during reproductive behaviours. Both sexes displayed a high ventilation rate during pre-copulatory aggression, followed by ventilatory arrest at the onset of seizing behaviours. By comparison, the heart rate of female crayfish increased from mating onset to dismounting behaviours. These results indicate that physiological measures may be a behaviourally relevant indicator for receptivity to crayfish sex pheromones.

This study shows that during pre-copulatory aggression ventilation rate and heart rate is increased, potentially to increase oxygen availability to meet high energy demands of fighting, or upcoming copulation. However when the male seizes the females and tried to turn her both male and female crayfish displayed periods of ventilatory arrest. Cessation of ventilatory and cardiac activity is associated with startling stimuli and has been interpreted as a mechanism of predator avoidance (McMahon 1999). Potential

predators, such as fish, can detect minute electrical disturbances in their environments and are therefore capable of detecting regular electrical pulses associated with cardiac/ventilatory activity (McMahon 1999). Brief cessation of these pulses can therefore reduce the risk of detection. This may be important during reproduction when crayfish are at higher risk of predation due to limitations in movement and restriction of the tail flip escape mechanism.

Prolonged cessation of ventilatory activity would be highly costly as following arrest efforts would need to be taken to compensate for oxygen deprivation (increased ventilation, increased cardiac activity). This would add to the overall 'cost' of mating, which is generally believed to be higher in females in comparison to males due to higher gamete investment (Trivers 1972). Due to the small sample size in our experiment it is difficult to determine if there are sex-specific differences in physiological responses during mating. More experiments would need to be performed to conclusively determine if there are any asymmetries in the physiological costs of mating for male and female crayfish.

During reproductive behaviours crayfish communicate using urinary pheromones, with male crayfish assessing female receptivity during pre-copulatory aggression (see chapter 3). This study correlated physiological measures to observable reproductive behaviours but did not assess the effect of chemical communication. Previous work in this thesis has shown that males reduce urine release during mating, whilst females release primarily during pre-copulatory aggression (see chapter 3). During urinary signalling ventilatory activity increases to produce gill currents, which aid odour delivery (see chapter 6). Therefore during precopulatory aggression it is expected that males have a slower ventilation rate, in comparison to females, and in relation to their ventilatory activity during male combats. Improvements to the sample size of this study and combination with the visualisation of urine release will help to show how ventilatory activity changes when crayfish are engaged in different social behaviours and when chemical signalling.

This preliminary study has demonstrated that ventilatory and cardiac rates are modified by crayfish during reproductive behaviours. This indicates that physiological measures can form the basis of a novel assay to be combined with purification techniques for pheromone identification. Previous experiments have shown that exposing an individual

crayfish to conspecific chemical cues can cause physiological changes, such as increased heart rate (Li et al. 2000). Systematic increases in ventilation rate have been used successfully as an assay for steroid detection by male round gobies, *Neogobius melanostomus* (Murphy & Stacey 2002). Future studies therefore need to focus on assay development and ensuring that controlled stimulation of male crayfish to female odours result in reliable and biologically relevant physiological responses (see chapter 7).

Chapter 6

Ventilatory measures in crayfish during social interactions: the effects of social dominance and urinary signalling

6.1 Introduction

Animals make behavioural decisions based on the continual evaluation of mechanical, visual and chemical cues in their environment. To understand how animals perceive these cues research has predominantly focused on observing changes in behavioural movements. Therefore when an animal does not make significant behavioural changes it can be classed as being unresponsive to an introduced stimulus. However studies of physiological parameters have shown dramatic changes in cardiac and ventilatory activity can occur in the absence of obvious external behaviours (McMahon 1999; Li et al. 2000; Schapker et al. 2002).

Alterations of internal physiology of decapod crustaceans have been found during social interactions. During fights with conspecifics the ventilation and heart rate of crayfish (*Procambarus clarkii*) has been shown to increase during visual threat displays and aggressive behaviours in comparison to neutral behaviours (Schapker et al. 2002). An adjustment of ventilatory and/or cardiac activity has the potential to prime the individual's circulatory system with oxygen and can help give crayfish a competitive edge during agonistic behaviours.

The ventilation rate of most decapod crustaceans can be recorded by measuring the rhythmic beating of the scaphognathites. These are paddle like appendages which oscillate in a narrow channel at the anterior end of the gill cavity and move water into the branchial chamber and across the gills (Vogt 2002). Scaphognathite movements have been found to play an important role in the signalling behaviour of astacid crayfish (Breithaupt & Eger 2002). It is extremely important for aquatic animals to disperse chemical signals via self generated currents, since molecular diffusion is extremely slow (Atema 1995). Observations of astacid crayfish, *Astacus leptodactylus*, have shown that forward directed gill currents carry urinary signals towards their opponent (Breithaupt & Eger 2002). When releasing urine, the fan organs (multi-segmental flagella of the mouthparts (maxillipeds) with feathered setae on the distal part) remain inactive and are not thought to play a role in distributing signals. By comparison, the fan organs are actively used by cambarid crayfish, such as *P. clarkii*, in conjunction with urine release (Breithaupt 2001; Breithaupt & Eger 2002). Previous studies have focused on the ventilatory activity of crayfish during agonistic interactions, however changes in ventilation rate during urinary signalling still needs investigation. An increase in ventilatory activity would indicate the production of gill currents for signal delivery.

The development of non-invasive measurement techniques has facilitated the recording of ventilation rate of decapods during a range of social situations (Depledge & Andersen 1990). To assess whether ventilatory activity is correlated with signal release this study aims to combine recordings of ventilation rate with the visualisation of urine release. In addition, the potential for crayfish to use multimodal signalling (Hebets & Papaj 2005), via both chemical and hydrodynamic signals, will be assessed. Multimodal signals have been found in the snapping shrimp, *Alpheus heterochaelis*, which produce fast and focused anteriorly directed gill currents in conjunction with chemical signalling (Herberholz & Schmitz 2001). These currents and water jets provide a powerful hydrodynamic tool in conspecific signalling.

This study also hopes to evaluate whether measures of ventilation rate could form the basis of an effective assay for identifying the chemical components of crayfish dominance pheromones. Physiological assays using changes in ventilatory activity of round gobies, *Neogobius melanostomus*, have provided a useful tool in assessing male responses to female steroidal pheromones (Murphy & Stacey 2002). Chemical identification of dominance odours would give insights into the mechanism of hierarchy formation and help to indicate whether these odours are important in female mate choice of male partners. Ventilation rate was studied for both signal and red swamp crayfish to draw comparisons between signalling systems of astacid and cambarid crayfish. It is expected that ventilation rate will increase during agonistic interactions and urinary signalling due to increased energy demands and the production of gill currents during fights.

6.2 Materials and methods

6.2.1 Animal maintenance

Adult red swamp crayfish (*P. clarkii*) were obtained in June 2006 from a crayfish supplier (Sunbeam Aquarium Ltd) having been trapped in China and imported from Singapore. Animals were left for two weeks in the lab to recover from shipment prior to experimental use.

Animals were maintained in a recirculating freshwater system containing six tanks (45 x 60 x 30cm) with a carbon filter (Pozzani Pure Water PLC, UK) and a UV filter (Lotus Water Garden Products, UK). Dividers were placed in the tanks which separated

crayfish physically but not visually or chemically. This was to prevent aggressive encounters within the tank which can lead to autotomy of limbs (pers. obs). A maximum of 12 crayfish were kept in each tank. Animals were maintained at 21°C with a light regime at 14:10 h light: dark.

Adult signal crayfish (*Pacifastacus leniusculus*) were obtained in Aug 2007 from a crayfish dealer (Chris Campbell, North Dorset) having been trapped in a local lake. Animals were maintained at 10°C, 10:14 h light: dark cycle. Animals were housed in an identical unit to that used for *P. clarkii*, although crayfish were not physically separated by dividers as individuals showed lower levels of aggression. A maximum of 25 animals were kept in each tank.

Crayfish were fed twice weekly, including on the morning of the experiment, with commercially available, defrosted prawns.

6.2.2 Agonistic interactions

To study ventilation rates during agonistic encounters we used 30 intermoult male signal crayfish (mean ± SE carapace size of 37.4 ± 0.59 mm, mass 36.6 ± 1.3 g) and 30 form I (sexually receptive) male red swamp crayfish (mean ± SE carapace size of 37.4 ± 0.59 mm, mass 36.6 ± 1.3 g), all with intact appendages.

Red swamp crayfish interactions were carried out in Jun – Jul 2006 whilst signal crayfish fights took place in Sept – Nov 2007. One week prior to experiments animals were size matched (within 5% carapace length and weight) and isolated in 3 l containers (24 x 17.5 x 8 cm). Crayfish were blindfolded 24 hrs prior to interactions by wrapping opaque plastic (1 x 4 cm) around the rostrum and eyestalks and securing excess material to the carapace using cyanoacrylate glue. This prevented potential visual disturbances from the ventilation recording equipment (wires and sensors). Each opponent was numbered using white corrective fluid as a visual marker on the carapace for identification during analysis.

Interactions took place in a glass aquarium (40 x 20 x 20 cm) which was adapted for filming by covering the walls with black opaque lining. Light from a 150 W slide projector (Reflecta Diamator AF, Germany) was reflected into the tank by a mirror (44 x 20 cm). Interactions were filmed from a front view only (Panasonic Digital

Camcorder, NV-95180). Following each experiment equipment was washed thoroughly using carbon filtered water.

Interactions started after a 10 min acclimatisation period, where animals were physically and chemically isolated by an opaque divider. Fights were recorded for a minimum of 15 minutes and filming stopped 5 minutes after a clear winner was established.

6.2.3 Ventilation rate recording

The ventilation rates of male crayfish were monitored using a non-invasive optoelectronic technique (Depledge & Andersen, 1990). One hour prior to the start of the fight an infrared sensor (SG-2BC, optosensor IR, Farnell, USA) was attached to the carapace over the right anterior branchial region of the crayfish using cyanoacrylate glue. The sensor is comprised of a light emitting diode (950 nm) and a phototransistor detector. The phototransistor could detect the changes in infrared light intensity scattered as a result of scaphognathite activity. This signal was filtered and amplified using a two channel amplifier (Dept. of Engineering, University of Hull) and transferred to a computer using a PowerLab AD converter (type ML880, ADInstruments). Scaphognathite beats were measured in mV and analysed using Chart 5.5 software (ADInstruments), which calculated an instantaneous ventilation rate (BPM).

Ventilation rate was recorded for the 10 min acclimation period and the duration of the agonistic interaction. If an unusual behaviour was performed or a disturbance occurred in the room it was noted on the Chart file and the section of influenced ventilation rate was not analysed.

6.2.4 Urine visualisation procedure in *P. clarkii*

To study changes in ventilation rate in relation to signalling behaviour red swamp crayfish were injected with the dye, fluorescein, to visualise urine release. In accordance with the methods developed by Breithaupt & Eger (2002), a 0.3 % sodium fluorescein solution (dose 9-10 $\mu g\ g^{-1}$ body mass) was injected into the pericardium region of crayfish 3-4 hrs prior to experiments using a 250 µl syringe (Hamilton, Switzerland) and a 45-gauge needle (Microlance, Ireland). After injection the hole was sealed using plasticine and tape to avoid haemolymph loss. The technique was successful in visualising urine in all individuals (N = 30). For interactions between red swamp crayfish the release of stained urine was recorded for both individuals during the fight.

6.2.5 Behavioural analysis

Filmed interactions were digitised and subsequently analysed using a behavioural software package (The Observer 5.0, Noldus Inc) which allowed behaviours to be scored on a continuous time scale.

Fights were analysed until the dominance status of opponents had been established and there was a clear winner (dominant male) and loser (subordinate male). For each experiment the subordinate male would determine when the fight was over by displaying submissive behaviours (fleeing, avoidance) and not re-engaging in highly aggressive behaviours (claw boxing, tearing, ripping) for at least 5 min. Several fight bouts could occur before the status was decided.

A mean ventilation rate (BPM) was calculated for the different behavioural categories displayed over the total fight duration. Behavioural categories were defined as (i) Isolation; the one min section prior to when interactions began, when animals were in isolation; (ii) Non-contact behaviours; when animals displayed initiation or threat display behaviours; (iii) Low aggression fighting; animals use their chelae to push or box their opponent, no grasping (iv) High aggression fighting; animals used their chelae to grasp onto their opponents body or displayed unrestrained levels of aggression (claw tearing, ripping); (v) Urine release; crayfish released stained urine (red swamp crayfish only).

6.2.6 Ventilation rate analysis

Ventilation rate was measured for different behaviours by counting the number of scaphognathite beats and dividing the total by the duration of the behaviour (beats per min, BPM).

6.2.7 Statistical Analysis

Prior to parametric analysis all data was tested for normality (Kolmogorov-Smirnov test). A two-way repeated measures ANOVA (within subject factor – behaviour; between subject factor – dominance) was used to analyse if there was a significant difference in the ventilation rate of dominant and subordinate crayfish during different behaviours. However, this analysis could not include ventilation rate during urine release as only six subordinate red swamp crayfish released urine during fights. In order

to analyse whether ventilation rate increased during urine release a separate one-way repeated measure ANOVA was performed to assess if red swamp crayfish increased their ventilatory activity whilst releasing urine, in comparison to other behaviours. For each ANOVA where a significant difference was found post hoc analysis was performed using a Tukey's HSD test.

To assess the change in ventilation rate during aggressive behaviours relative to the ventilation rate when animals were in isolation, a standardised ventilation rate was calculated. This eliminated intrinsic differences between individual crayfish. A standardised ventilation rate of one indicates no change from the ventilation rate recorded when animals are in isolation. The standardised ventilation rate was only calculated for times when animals displayed high aggression fighting as this was when the differences between the ventilation rate of dominant and subordinate crayfish were highest. Standardised ventilation rates were compared for dominant and subordinate crayfish using a paired t test (data was checked for normality; Kolmorov – Smirnov test $p > 0.05$), a separate test was performed for red swamp crayfish and signal crayfish.

6.3 Results

The ventilation rate of male red swamp crayfish and male signal crayfish increased in relation to the intensity of the aggressive interaction (figure 6.1, figure 6.2; table 6.1); with the mean ventilation rate (BPM) being significantly higher during aggressive behaviours in relation to when animals were in isolation (two-way repeated measures ANOVAs; red swamp crayfish $F_{3,28} = 31.24$, $p < 0.001$; signal crayfish $F_{3,28} = 31.49$, $p < 0.001$). There was no effect of dominance (two-way repeated measures ANOVA; red swamp crayfish $p = 0.79$; signal crayfish $p = 0.28$), and no interaction effect between dominance and behaviour (two-way repeated measures ANOVA; red swamp crayfish $p = 0.07$; signal crayfish $p = 0.20$) on the ventilatory activity of either species.

Table 6.1: Two-way repeated measures ANOVA for ventilation rate of (A) signal crayfish and (B) red swamp crayfish during agonistic behaviours. Within subject factor – behaviour; between subject factor – dominance).

A

Factor	df	F	p
Behaviour	3	31.49	< 0.001
Dominance	1	1.19	0.28
Interaction	3	1.57	0.20
Total	84		

B

Factor	df	F	p
Behaviour	3	31.24	< 0.001
Dominance	1	0.07	0.79
Interaction	3	2.42	0.07
Total	84		

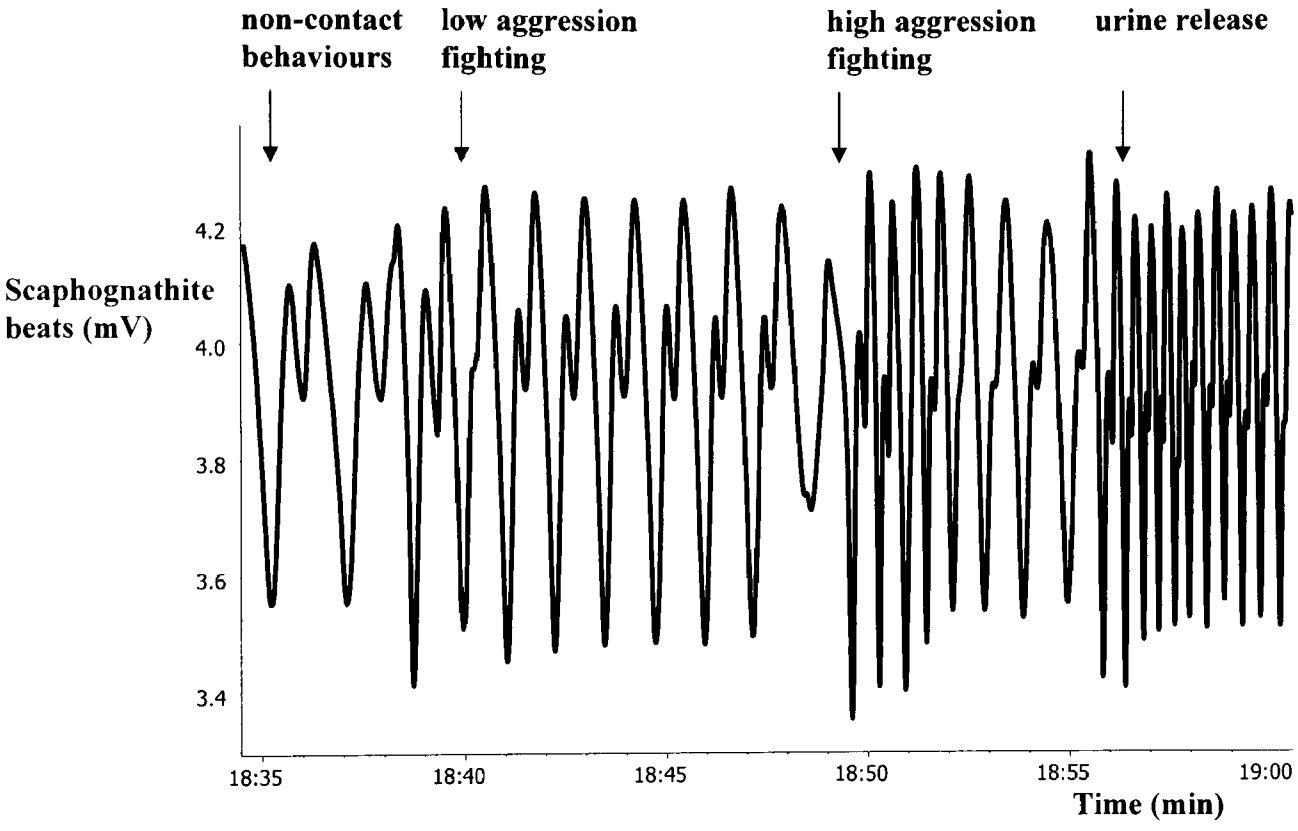

Figure 6.1: Example of the ventilatory activity of an adult male red swamp crayfish during aggressive fighting. Ventilation rate increases in relation to the level of agonistic behaviours.

In comparison to subordinate crayfish, dominant males showed a stronger increase of ventilation rate during high aggression fighting, relative to the ventilation rate when in isolation (*P. clarkii*; $t = 2.231$; $p < 0.05$; *P. leniusculus*; $t = 3.067$; $p < 0.01$; Paired t-tests).

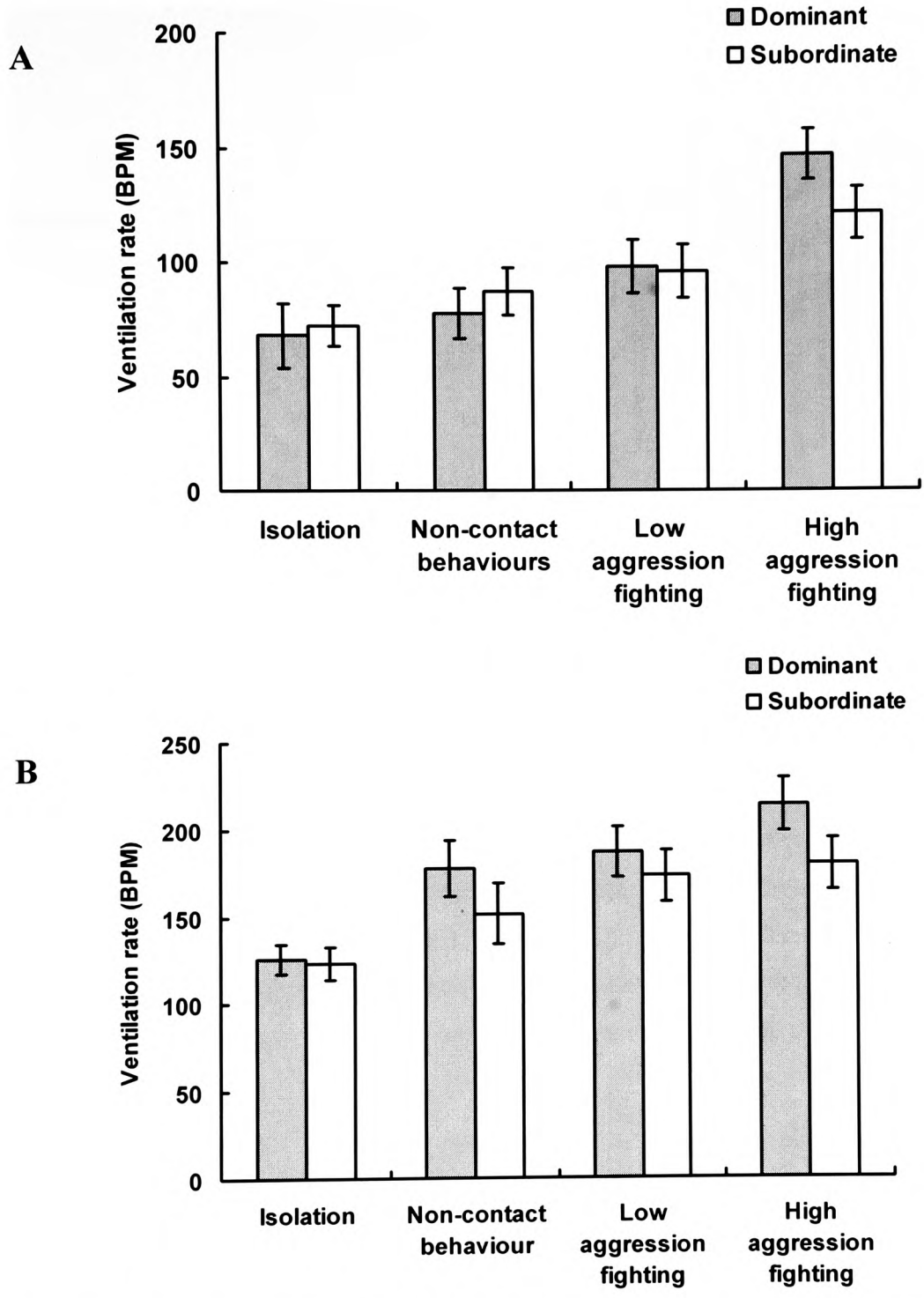

Figure 6.2: The mean ventilation rate of crayfish when displaying different aggressive behaviours (A) *P. clarkii* (B) *P. leniusculus*. Ventilation rate increased in relation to the intensity of the interaction. There was no effect of dominance on ventilation rate.

Male crayfish occasionally displayed a brief cessation of scaphognathite beating during aggressive behaviours. This was usually followed by a period of increased ventilation rate (figure 6.3).

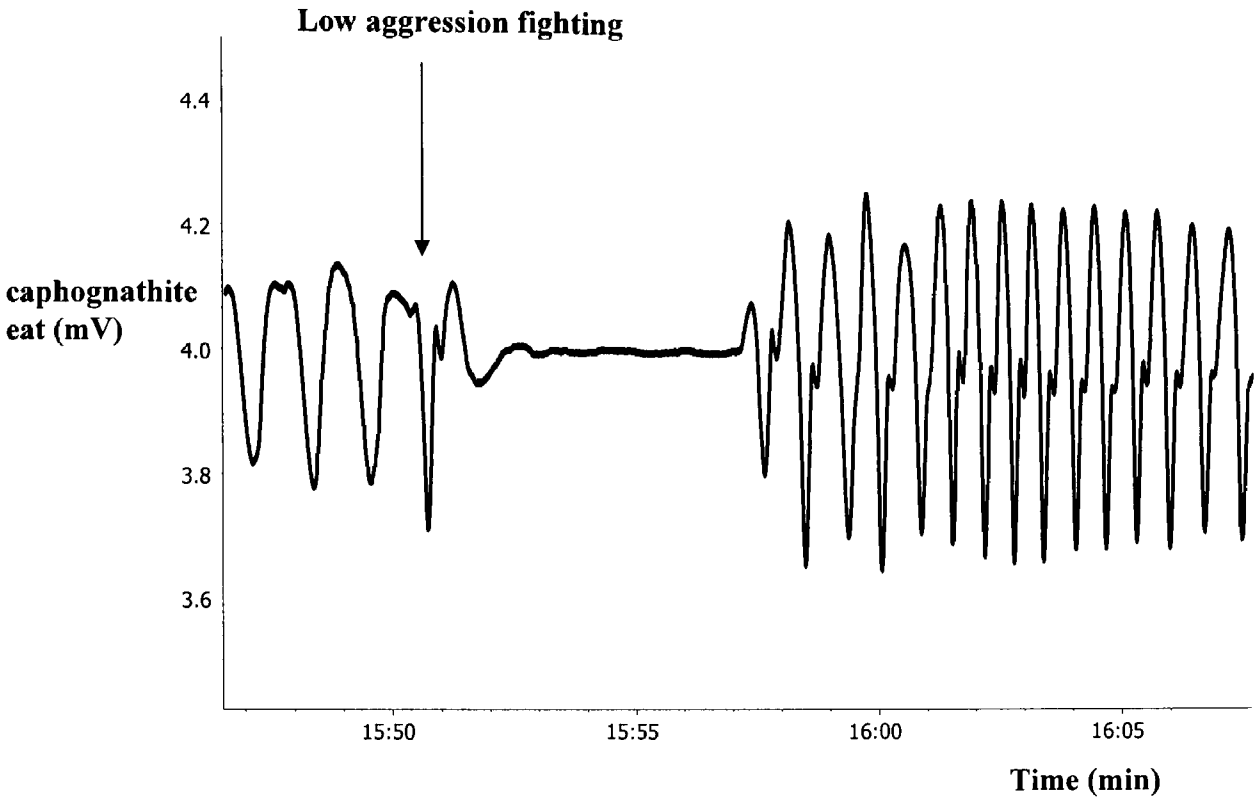

Figure 6.3: Example of the scaphognathite activity of an adult male signal crayfish at the onset of aggressive fighting. A brief cessation of ventilatory activity occurred at the start of aggressive behaviours, followed by a period of increased ventilation rate.

Red swamp crayfish showed elevated levels of ventilatory activity whilst releasing urinary signals, in addition to when displaying highly aggressive behaviours (figure 6.4; one-way repeated measures ANOVA $F_{4,20} = 42.15$, $p < 0.01$). Ventilation rate during urine release was significantly increased from levels displayed during low aggression fighting, non-contact behaviours or when animals were in isolation (figure 6.5).

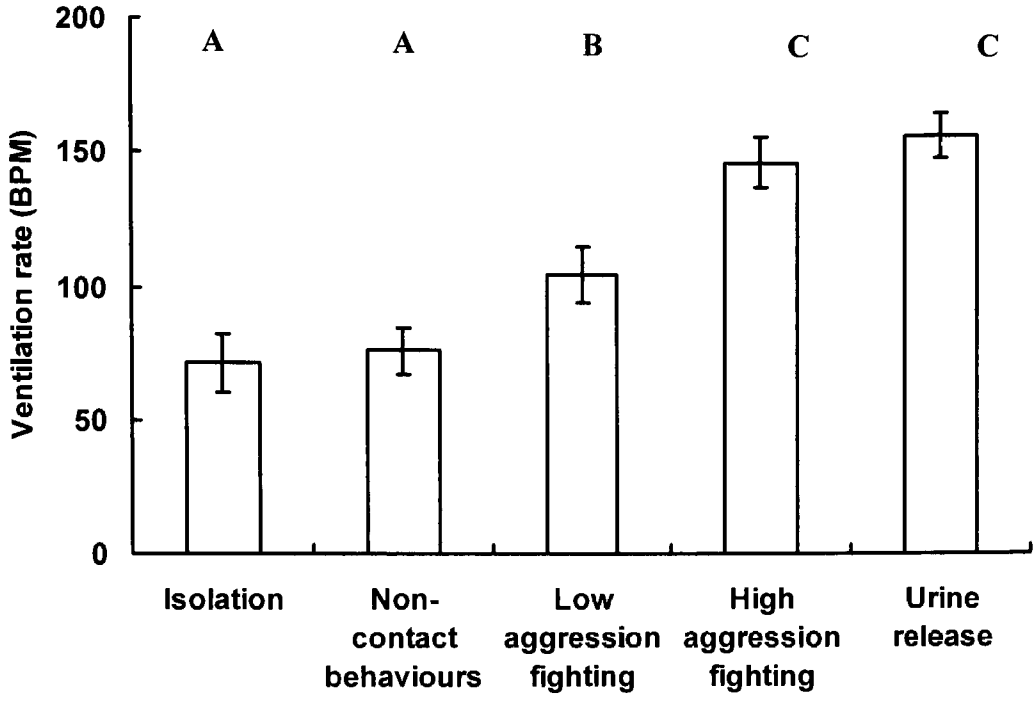

Figure 6.4: The mean ventilation rate of male red swamp crayfish which released urine during fights (N = 21). Ventilation rate increased as a result of increasing intensity of fighting, with the highest level of ventilatory activity being associated with times when urine was being released. Bars not connected by the same letter are significantly different ($p < 0.05$, Tukey's HSD post-hoc test).

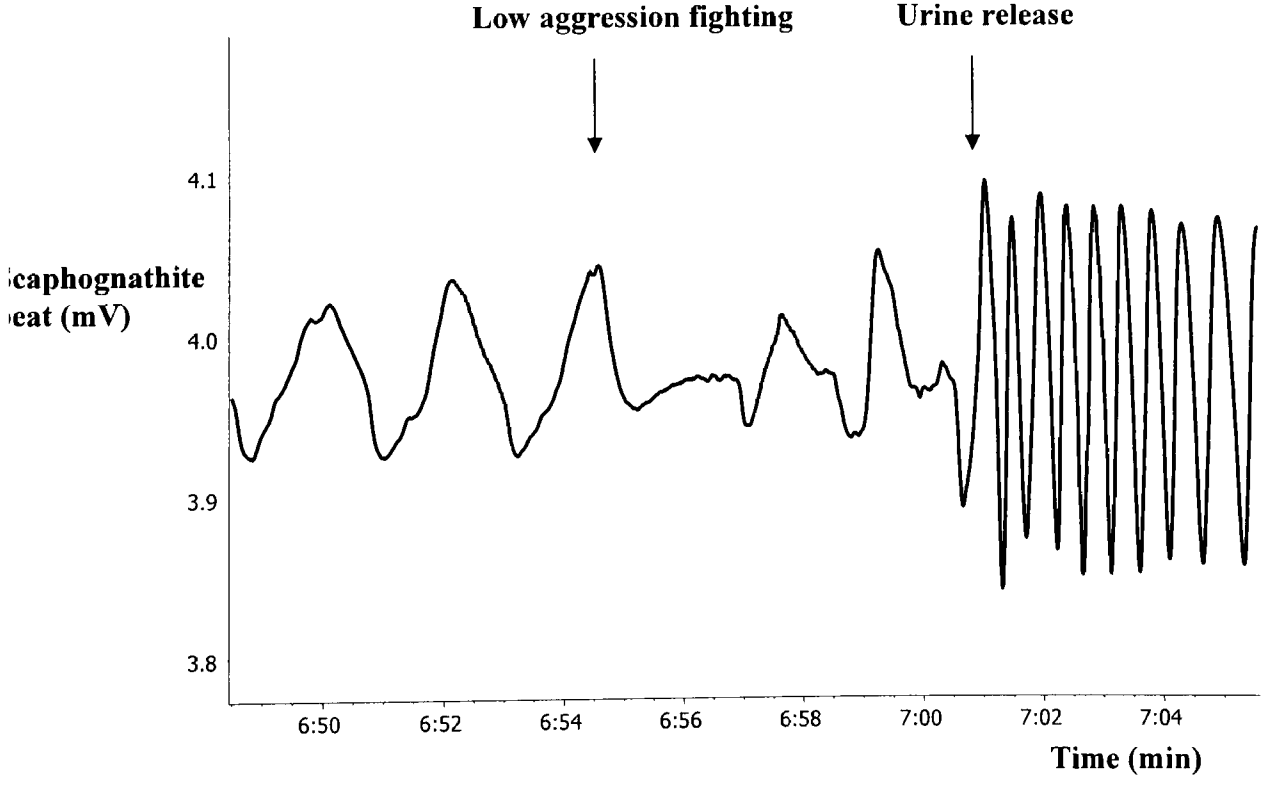

Figure 6.5: Example of the scaphognathite activity of an adult male red swamp crayfish. Ventilation rate is increased at the onset of urinary signalling.

6.4 Discussion

During agonistic interactions the ventilation rate of male crayfish (*P. leniusculus*, *P. clarkii*) increased with fight intensity. During highly aggressive behaviours dominant males were found to increase their ventilation rate to a greater extent (relative to when animals were in isolation) than subordinate males. In addition ventilatory activity was significantly increased during urinary signalling by red swamp crayfish. Alterations in physiological parameters therefore appear to provide a sensitive indicator for signal delivery in comparison to behavioural observations.

This study demonstrates that crayfish are capable of rapidly responding to external stimulation and changing social conditions through altered ventilatory activity. During fights crayfish increased ventilation rate in relation to the escalation of aggressive behaviours. Increases in ventilatory activity complement increases in cardiac activity (see Schapker et al 2002) and helps to prime the circulatory system with oxygen, ready for the energetic demands of the fight. This kind of response could be compared to those displayed by vertebrates, which are mediated by the autonomic nervous system, such as the fear fight or flight responses (Nicholls et al. 2001).

Winners of agonistic interactions altered their ventilation rate to a greater extent than subordinate opponents, which potentially increases the oxygen availability to dominant males. Energy expenditure during a fight is likely to be related to oxygen consumption and scaphognathite activity (Smith & Taylor 1993) and therefore increases in ventilation rate are costly. Measurements of lobsters engaged in fighting have shown that, similarly to the results presented here, winning lobsters are capable of adjusting physiological parameters (heart rate) to a greater extent than losers (Hernández-Falcón et al 2005). It is possible that losers are incapable of adjusting their physiological state to as greater extent as winners, or they may not have the energy reserves to pay the extra costs of an increased ventilatory/cardiac activity. Alternatively, modulation of physiological parameters may be effected by hormonal changes brought about by determination of social status brought about by contest outcome (Hernández-Falcón et al 2005). Being able to rapidly alter cardiac and ventilatory activity may give winners a competitive advantage over subordinates and result in an increased access to limiting resources, such as food and mates.

This study has shown that red swamp crayfish increase their ventilatory activity whilst releasing agonistic urinary signals. This suggests that forward gill currents are created to distribute signals, potentially in conjunction with currents created by the fan organs (Breithaupt 2001; Breithaupt & Eger 2002). Self generated currents have previously been found to be important in signal delivery in crayfish, although not all of the currents generated were associated with urine release (Bergman et al. 2005). It is possible that these currents form part of a multimodal form of signalling with strength of hydrodynamic signals being as important as chemical signals; as has been found in snapping shrimp, *Alpheus heterochaelis* (Herberholz & Schmitz 2001). Self generated currents are important for a range of decapod crustaceans, with lobsters (*Homarus americanus*) being capable of producing water jets that can reach up to seven body lengths (Atema 1985). Being able to create currents to distribute signals should improve signal efficiency and reception by the receiving opponent. This could be equally important for species which inhabit still, turbid waters (such as *P. clarkii*) and for species inhabiting riverine habitats, where currents could deviate the direction of the odour signal away from the opponents chemoreceptors.

For the duration of this experiment crayfish were blindfolded. Despite the lack of visual stimuli crayfish were still capable of sensing their opponent's presence during non-contact behaviours. This highlights the importance of mechano-sensory and chemical cues during social situations. Blind crayfish have been found to respond physiologically (increased heart rate) to chemical and/or hydrodynamic signals of conspecific opponents prior to any physical contact and fighting (Li et al. 2000). Being able to detect an opponent prior to fighting onset can be an advantage when priming physiological responses.

Due to the clear physiological responses shown in this study (increased ventilation rate during aggressive behaviours) changes in ventilatory activity may provide a useful index to indicate the receptivity of male crayfish to chemical cues released by conspecifics. A physiological index can be particularly useful as animals may not show obvious and distinct behavioural responses to subtle changes in chemical cues. When designing a physiological assay to test receptivity it is important to ensure that natural responses (i.e. increased ventilation rate) are observed following controlled exposure to exogenous male dominance odours. If reliable responses can be achieved physiological measures should be useful in an assay to be coupled with analytical chemistry

techniques to determine the active chemical components of urinary dominance cues. This would help to unlock information about how crustaceans form dominance hierarchies.

In conclusion, this experiment has shown that crayfish are capable of rapidly adjusting physiological parameters to changing social situations. Ventilation rate increased in relation to fight intensity and urinary signalling. This has shown that red swamp crayfish utilise gill currents to disperse agonistic pheromones to opponent crayfish. Future experiments will demonstrate whether other species distribute currents in this way.

Chapter 7

Development of behavioural and physiological assays to assess discrimination of male and female odours in crayfish, *Pacifastacus leniusculus*

The majority of work in this chapter has been published in the following paper;

Berry, F. & Breithaupt, T. 2008 Development of behavioural and physiological assays to assess discrimination of male and female odours in crayfish, *Pacifastacus leniusculus*. Behaviour: 145, 1427 – 1446.

7.1 Introduction

Since the first mate attractant molecule was discovered for the silkworm moth, *Bombyx mori* (Butenandt et al. 1959) a tremendous research effort has gone into unravelling the molecular structures of insect chemical signals, mainly due to their application in integrated pest management. In contrast there has been limited success in identifying the chemical structure of water borne cues, which are harder to detect and measure than their visual and acoustic counterparts. Identified aquatic sex pheromones include those of the ragworm, *Nereis succinea* (Hardege et al. 2004), the shore crab, *Carcinus maenas* (Bublitz 2007) and a number of fish species including the goldfish, *Carassius auratus* (Stacey & Sorensen 2005).

Studies into the chemical nature of pheromones require interdisciplinary efforts including chemical analysis and biological assays. Bioassay design needs to be governed by two essential objectives: ecological relevance and methodological simplicity. A bioassay used in sex pheromone identification should evoke a specific sexual response in the subject animal following chemical stimulation. For the development of behavioural assays it is therefore imperative to understand the natural context of chemical communication including signal delivery and receiver response (Dunham 1978; Wyatt 2002). Initial attempts to identify pheromones were based solely on behavioural assays where responses were simple and reproducible (Karlson & Butenandt 1959). Behavioural bioassays have since been combined with physiological measures, such as electro-antennograms in insects (Chapman 2000) and electro-olfactograms in fish (Li et al. 2002; Stacey & Sorensen 2005), to aid pheromonal identification. Physiological assays are generally quicker and result in more replicates in a set timeframe than behavioural assays which, in contrast, provide more specific responses. At present no physiological assays have been employed to aid identification of crustacean pheromones, with the most recent crustacean sex pheromone being identified using behavioural bioassay alone (Bublitz 2007; *C. maenas* sex pheromone).

There is considerable interest in identifying the sex pheromone of the invasive crayfish *Pacifastacus leniusculus*. Signal crayfish are endemic to North America but were introduced to Europe in the 1960's, with populations establishing in the UK from the late 1970's (Souty-Grosset et al. 2006). They are now considered virulent pests throughout Europe, directly contributing to declines in indigenous crayfish populations (Nystrom 2002; Souty-Grosset et al. 2006). Pheromone baits have been proposed as a

way to increase species-specificity and efficiency of trapping regimes during the breeding season (Stebbing et al. 2004). The use of pheromone baits to lure and trap individuals have been extremely successful in trapping insect pests in terrestrial systems (Agosta 1992) but little is known about their application in aquatic environments (Corkum 2004). An exception is the integrated sea lamprey (*Petromyson marinus*) management scheme in the Great Lakes of North America which is currently in its initial stages following recent identification of the pheromone's chemical components (Li et al. 2003; Sorensen & Vrieze 2003).

Attempts to identify the female sex pheromone of signal crayfish have been hindered by the lack of suitable bioassay. Previous studies on crayfish sex pheromones have yielded contradictory results, with some studies indicating male crayfish can distinguish conspecific male from female odours (Ameyaw-Akumfi & Hazlett 1975; Hazlett 1985; Dunham & Oh 1990) whilst others have reported no discrimination (Thorp & Ammerman 1978; Itagaki & Thorp 1981). Contradictions in bioassay results have been attributed to the difficulties of identifying specific sexual responses and recognising crayfish discrimination of sex-specific odours (Dunham 1978; Rose 1982; Rose 1984; Bechler 1995). Recent bioassay designs have used non-sexual behaviours to show differential male responses (activity, airstone handling) to water of mature female versus juvenile females, suggesting recognition of a mature female cue (Stebbing et al. 2003a; Belanger & Moore 2006). However the sex-specificity of responses was not tested.

The mating behaviour of crayfish has been studied in several crayfish species (Mason 1970; Villanelli & Gherardi 1998) with that for *P. leniusculus* described as having seven distinct stages (see Stebbing et al., 2003 and chapter three for full description). The behaviours of seizing, turning and mounting are unambiguous sexual behaviours which could form the basis of the behavioural bioassay. The use of fluorescein as a visual marker has shown urine to be the source of pheromone signals (chapter three; Breithaupt & Eger 2002; Simon & Moore 2007). Urine is released into the gill currents and transported towards the opponent crayfish (Breithaupt & Eger 2002), with females releasing urine at the onset of reproductive behaviours (see chapter three).

This study aims to develop behavioural and physiological assays suitable for subsequent identification of crayfish sex pheromones. We use current knowledge of crayfish mating

behaviour to develop a behavioural assay based on unambiguous sexual behaviours of male crayfish towards a dummy female. If the male tries to differentially seize, turn or mount the dummy, following the introduction of male and female odours the bioassay could be employed in efforts to chemically identify the sex pheromone.

In addition to the behavioural assay, physiological measures (heart rate, ventilation rate) are assessed as a quick measure of odour discrimination. Physiological methods such as electro-olfactograms (Li et al. 2002) and ventilation rate recordings (Murphy et al. 2001; Belanger et al. 2007) have previously been used to identify fish sex pheromones. Studies of physiological measures (heart rate, ventilation rate) during reproductive behaviours show that crayfish can respond through arrest in activity followed by an increased in rate (chapter five). This indicates physiological measures could provide a quick and reliable bioassay test for signal crayfish. Two physiological assays based on changes in heart rate and changes in ventilation rate are tested which may complement the behavioural assay by providing a quick assessment of the male's pheromone recognition. It is hoped that ventilation rate assays could utilise the role that gill currents play in the signalling behaviour of crayfish. Observations during agonistic interactions between narrow clawed crayfish (*Astacus leptodactylus*) have shown that forward directed gill currents are produced which carry urinary signals towards their opponent (Breithaupt & Eger 2002). Gill currents are created by the beating of the scaphognathites (the exopodites of the second maxillae) which facilitates gas exchange and results in a forward projection of water below the antenna (Vogt 2002). The active use of gill currents to disperse signals is reflected by the increase in ventilation rate by crayfish when releasing urine (see chapter six).

7.2 Materials and methods

7.2.1 Animal holding conditions

Adult crayfish (*P. leniusculus*) were obtained in September 2006 and 2007 from a crayfish dealer (Chris Campbell, North Dorset) having been trapped in a local lake, just before breeding season. For at least two weeks prior to the experiments animals were separated by sex and kept in a communal holding tank (91.5 x 30 x 30 cm). A maximum of 25 animals were kept in the tank and males were physically but not chemically separated from females. Temperature in the holding tanks was gradually reduced from 18°C to 10°C and the light cycle was adjusted to 10:14h light:dark, mimicking field

conditions in the breeding season (Sept – Nov). Crayfish were fed twice weekly on commercially available defrosted prawns.

One week prior to the bioassay trials male crayfish were isolated in separate 3 l PVC containers (24 x 17.5 x 8 cm). Crayfish were blindfolded 24 hr prior to the experiment by wrapping a thin film of opaque plastic (1 cm x 4 cm) around the eyestalks and rostrum and securing excess material to the carapace using cyanoacrylate glue.

7.2.2 Urine and conditioned water collection

Urine samples were collected from mature male and female crayfish within the breeding season. A micropipette tip (1-10 µl, Fisherbrand) was inserted into the opening of the nephropore which caused urine to flow out from the bladder, to be collected by suction into Teflon tubing (1.5 mm diameter) and a 1.5 ml collection vial (method modified from Bamber & Naylor 1997). Urine samples tested were 0.5 ml pooled samples, collected less than one hour prior to the experiment. Samples were pooled on average from three individual crayfish with approximately 50 µl - 250 µl from each individual. Different donor animals contributed to urine samples for each experiment.

Water was conditioned by keeping individual crayfish in 300 ml filtered water (12 x 12 x 6 cm) for 24 hr. We used an individual crayfish, instead of multiple animals like in previous studies (Stebbing et al. 2003a), to reduce the chance of stress or agonistic pheromones being released into the water. Control water was maintained in the same environment as conditioned water prior to experimental use. All water used in the experiment was filtered through a 25 cm pre-sediment filter followed by a 25 cm activated carbon filter (Pozzani Pure Water PLC, UK).

7.2.3 General bioassay procedure

Bioassay trials were carried out in a glass aquarium (30 x 20 x 20 cm) which was adapted for filming by covering three walls with black opaque lining and using a removable black velvet panel as flooring. The aquarium was lit using a 60 W desk lamp.

A dummy female was constructed from a female moult shell (carapace length 34 mm). The shell was dried in a posture representative of a receptive female (claws outstretched in front of the body, body lowered to the ground) and covered in two-component glue.

The underside of the shell was covered in aquarium silicon to increase durability. Fishing weights were imbedded in the dummy so its weight was comparable to an adult female crayfish (33.5 g). A hole was pierced into the bottom of the dummy through which a thin tube (0.6 mm diameter) was positioned at the level of the nephropores.

The dummy female was placed at the opposite end of the aquarium from the experimental animal. The male was physically separated from the dummy by an opaque divider for an acclimation period of 15 min. Prior to lifting the divider water was released from the dummy (10 ml min^{-1}) through the thin tube, representing the flow of gill water normally extruded from a live crayfish. One min after the divider was lifted the experimental stimulus (urine, conditioned water or filtered water) was introduced by replacing the water reservoir with a syringe containing the stimulus (0.5 ml urine, 10 ml water, and 10 ml conditioned water) mixed with fluorescein. Fluorescein was added to the stimuli to visualise when the male first encountered the odour plume. Once the stimulus was added the water reservoir was reconnected.

Following the experiment all equipment was washed fully using carbon filtered water. Each bioassay trial was given a code so the analyser did not know the identity of the released stimulus.

7.2.4 Behavioural bioassay

Eighty-seven intermoult male crayfish (mean ± SE post-orbital carapace length of 37.3 ± 0.73 mm, mass 33.7 ± 1.4 g) with intact appendages were used in the behavioural bioassay trials. Experiments were carried out within the breeding season in October 2006 and 2007. A separate male was used for each trial and was only exposed to one stimulus. Trials started as soon as the stimulus reached the male subject and were filmed (Sony Hi8, CCD_VX1E) from a side view for 10 min. Seven trials were discarded from analysis as animals either displayed feeding behaviours towards the dummy (N = 3), remained stationary for over 90% of the experimental period (N = 2), or climbed on or touched the dummy while trying to escape from the tank (indicated by "wall hugging" and lifting claws to water surface, N = 2).

Five different experimental stimuli were employed in the behavioural bioassay trials: female urine, female conditioned water, male urine, male conditioned water and a control of filtered water (N = 16 for each condition).

Filmed trials were digitised and then analysed using a behavioural software package (The Observer 5.0, Noldus Information Technology, Wageningen, The Netherlands) which allowed behaviours to be scored on a continuous time scale. Three behaviours were scored throughout the experiment: (i) 'mobile', the animal is moving around the tank (ii) 'stationary', the animal stays in one position in the tank and (iii) 'touching', the animal is in contact with the model. Furthermore, three distinct categories were identified within the touching behaviour: (i) 'touching with chelipeds', contact using only the claws to touch the dummy; (ii) 'touching using the rest of the body', contact with the female dummy by the pereopods or the telson and (iii) 'mounting', male attempted to seize (grabbing the rostrum), mount (climbing onto the back of the model) or turn (using the pereopods) the dummy female. These behaviours were combined into the single category 'mounting' as distinct turning and seizing attempts were rare (N = 2).

Data on the time spent mobile, stationary or touching the model and comparisons of specific touching behaviours were compared for each stimulus using a one-way ANOVA with LSD post hoc tests. Where parametric test assumptions were violated the non-parametric Kruskal-Wallis test was used with Mann-Whitney tests as post-hoc analysis.

The number of males which displayed mounting behaviours was recorded for each experimental stimulus. A Chi-square test (with Fisher's exact tests) was used to assess if significantly more male crayfish displayed 'mounting' behaviours when female odours had been released in comparison to male odours and the control.

7.2.5 Heartbeat assay

Forty-eight intermoult male crayfish (mean ± SE carapace size of 37.4 ± 0.59 mm, mass 36.6 ± 1.3 g) with intact appendages were used in the heartbeat assay trials. Experiments were carried out within the breeding season in October and November 2006. Males were only used in one trial and had not previously been used in the behavioural bioassay.

Heartbeats were monitored using a non-invasive optoelectronic technique (Depledge & Andersen 1990). One hour prior to the start of the assay trial an infrared sensor (A312-95 optosensor IR, Farnell, USA) was attached to the carapace over the cardiac region of the crayfish using cyanoacrylate glue. The sensor is comprised of a light emitting diode (950 nm) and a phototransistor detector. The phototransistor could detect the changes in infrared light intensity scattered as a result of the heart beating. This signal was filtered and amplified using a two channel amplifier (Dept. of Engineering, University of Hull) and transferred to a computer using a PowerLab AD converter (type ML880, ADInstruments). The heartbeat was measured in mV and analysed using Chart 5.5 Software (ADInstruments) which calculated an instantaneous heart rate (BPM, beats per min) and the periods (sec) between individual heartbeats.

Heartbeats were recorded during the 15 min acclimation period and for 15 min after the stimulus had reached the test animal. Animals were filmed (Sony Hi8, CCD_VX1E) for the duration of the experiment from a front view. If an unusual behaviour was performed or a disturbance occurred in the room it was noted on the Chart file and the section of influenced heart rate was not analysed.

Three experimental stimuli were employed in the bioassay trials: female urine, male urine and a control of filtered water ($N = 16$ for each condition).

To assess immediate responses (such as cardiac arrest) of male crayfish to the experimental stimulus, we compared the single heartbeat period post-stimulation to the preceding 20 periods. If the period of the post-stimulus heartbeat was outside the confidence limits (mean ± S.E) of the periods of the 20 preceding heartbeats the trial was scored as a positive response. A Chi-square analysis (with Fisher's exact tests) was used to compare results between male urine, female urine and the water control.

In addition we assessed changes in average heart rate as a result of stimulation. Heart rate (mean of a 10 sec section, BPM) was measured: (i) 5 min prior to the introduction of experimental stimulus (HRp), (ii) immediately after introduction (HR0), (iii) 5 min post-stimulation (HR5) and (iv) 10 min post-stimulation (HR10).

To eliminate intrinsic differences between individual males, heart rate was standardised by expressing it as a proportion of the pre-stimulus heart rate (i.e. heart rate at time 0 is

expressed as HR0/HRp). A standardised heart rate of one indicates no change from the pre-stimulus heart rate. Differences between standardised heart rate at time 0 (Δ HR0), 5 min (Δ HR5) and 10 min (Δ HR10) for each stimulus were compared using a Friedman test, with Wilcoxon signed-rank tests used for post hoc comparisons. The standardised heart rate was also compared between stimuli at 0 min, 5 min and 10 min post-stimulation using a Kruskal Wallis test.

7.2.6 Ventilation assay

Forty-eight intermoult male crayfish (mean ± SE carapace size of 37.65 ± 0.53 mm, mass 38.3 ± 1.3 g) with intact appendages were used in the ventilation assay trials. Experiments were carried out within the breeding season in October and November 2007.

The ventilation rate of crayfish was recorded by measuring scaphognathite beats, using the same non-invasive optoelectronic technique (Depledge & Andersen 1990), as used for heartbeat analysis. An infrared sensor (SG-2BC optosensor IR, Farnell, USA) was attached to the anterior of the right branchial chamber to detect scaphognathite beats. This sensor was smaller than that used for heartbeat analysis; reducing the potential hindrance of chelae movement. Scaphognathite beats were measured in mV and analysed using Chart 5.5 Software (ADInstruments), which calculated an instantaneous rate (BPM, beats per min) and the periods (sec) between individual beats.

The same analytical and statistical techniques were employed as used for heartbeat analysis.

7.3 Results

7.3.1 Behavioural bioassay

There was no significant difference between the mean time that male crayfish spent mobile (One-way ANOVA; $F_{4,75} = 0.539$, $p = 0.71$) or stationary (One-way ANOVA; $F_{4,75} = 2.137$, $p = 0.58$) after the release of each experimental stimulus. However males spent significantly less time touching the female dummy following the release of control water in comparison to all other stimuli (One-way ANOVA; $F_{4,75} = 3.771$, $p < 0.01$, LSD post hoc tests $p < 0.01$).

Significantly more males mounted the dummy following the release of female urine and female conditioned water (figure 7.1; Chi-square; $\chi^2 = 14.28$, df = 4, $p < 0.01$) in comparison to male conditioned water (Fisher's exact test; $p < 0.01$) and the control water (Fisher's exact test; $p < 0.05$).

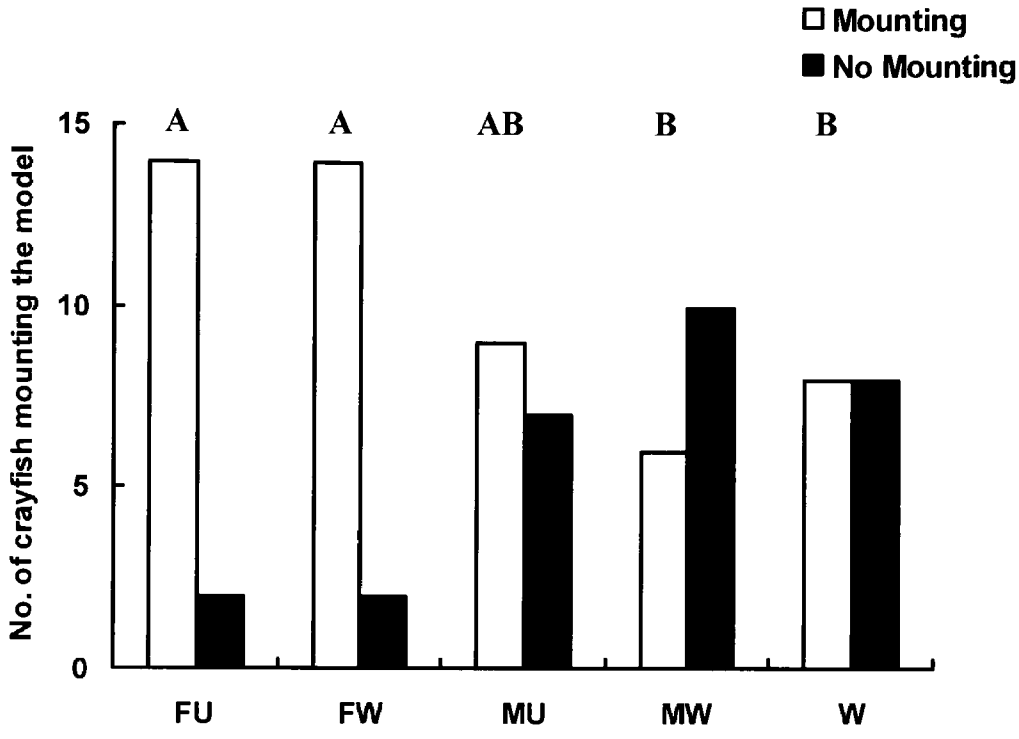

Figure 7.1: The number of male crayfish which mounted the female dummy (white bars) or displayed no mounting behaviours (black bars) following the introduction of different stimuli: FU, female urine; FW, female conditioned water; MU, male urine; MW, male conditioned water and W, water. $N = 16$ for each condition. Significantly more males mounted the dummy following the release of FU and FW in comparison to MW and W. Bars not connected by the same letter are significantly different ($p < 0.05$; Fisher's exact test).

Males spent significantly more time mounting the dummy following the release of female urine and female conditioned water in comparison to control water and male conditioned water (figure 7.2A; Kruskal Wallis; $\chi^2 = 23.664$, df = 4, $p < 0.001$; Mann-Whitney post hoc tests; $p < 0.01$). In addition, males mounted the dummy for significantly longer following the release of female urine in relation to male urine (Mann-Whitney post hoc test; $p < 0.05$).

There was no significant difference in the time males spent touching the dummy with the pereopods other than the chelae between all experimental stimuli (figure 7.2B. Kruskal Wallis; $\chi^2 = 8.709$, df = 4, $p = 0.07$). However males spent significantly less

time touching the dummy with their chelae following the release of control water (Kruskal Wallis; $\chi^2 = 14.383$, df = 4, $p < 0.01$) in comparison to other conditions (Mann Whitney post hoc tests; $p < 0.01$; figure 7.2B).

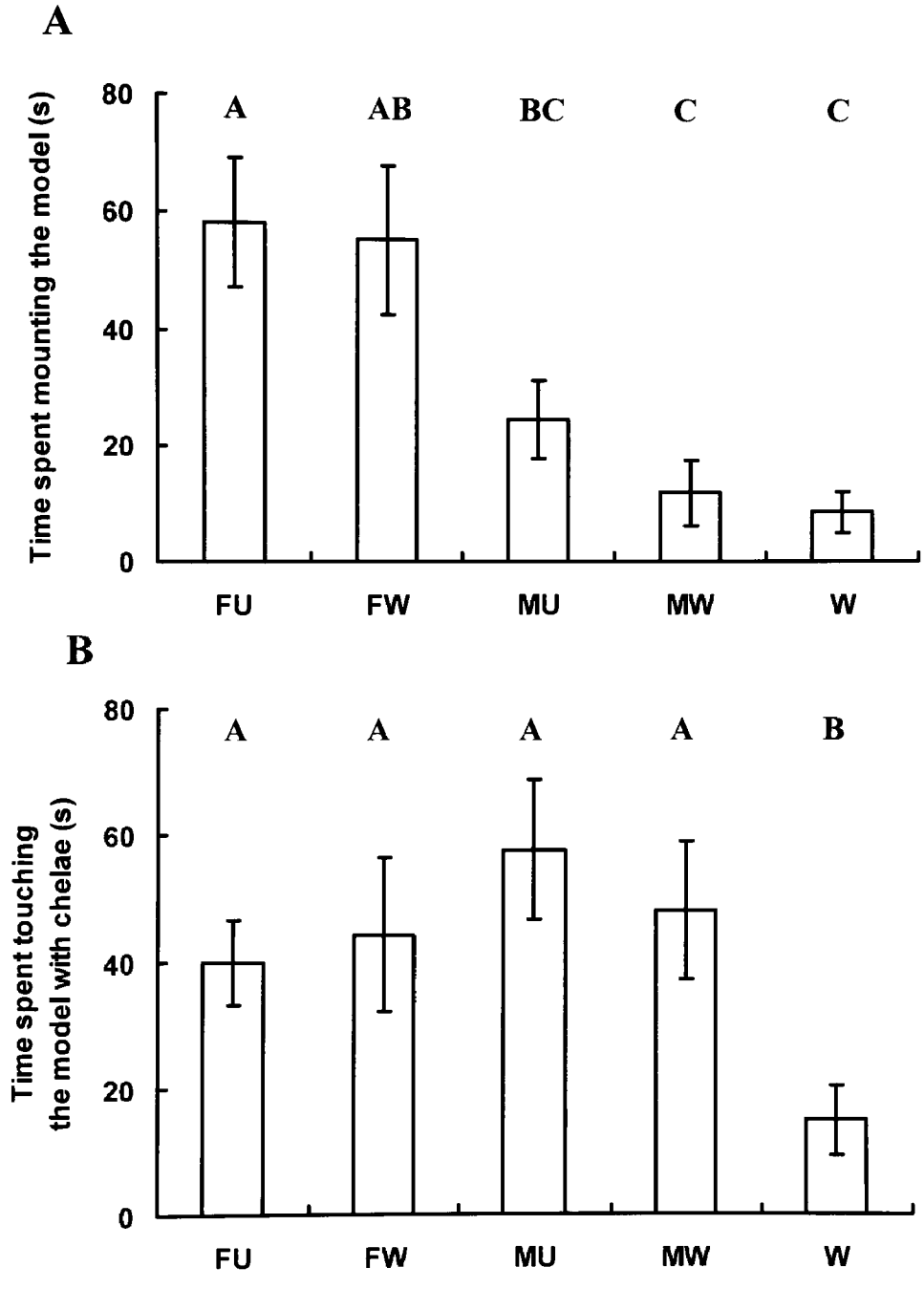

Figure 7.2: Timed behavioural responses of male crayfish to the female dummy following the introduction of different stimuli: FU, female urine; FW, female conditioned water; MU, male urine; MW, male conditioned water and W, water (N = 48). (A) The mean time male crayfish spent mounting the female dummy. Males spent significantly more time mounting the dummy following release of FU and FW in comparison to CW and MW. (B) The mean time male crayfish spent touching the

female dummy with their chelae. Values are mean times (sec) ± S.E. Bars not connected by the same letter are significantly different ($p < 0.01$; Mann-Whitney post hoc tests).

7.3.2 Heartbeat assay

Prior to stimulation the heartbeat period displayed by male crayfish ranged from 0.40-1.92 sec, post-stimulation the range grew to 0.41-9.48 sec. Upon introduction of the stimulus some crayfish reacted by displaying cardiac arrest for a few heartbeats before heart rate increased to a higher rate than pre-stimulus levels (figure 7.3). The longest cardiac arrest by a male crayfish lasted 9.48 sec following the introduction of female urine.

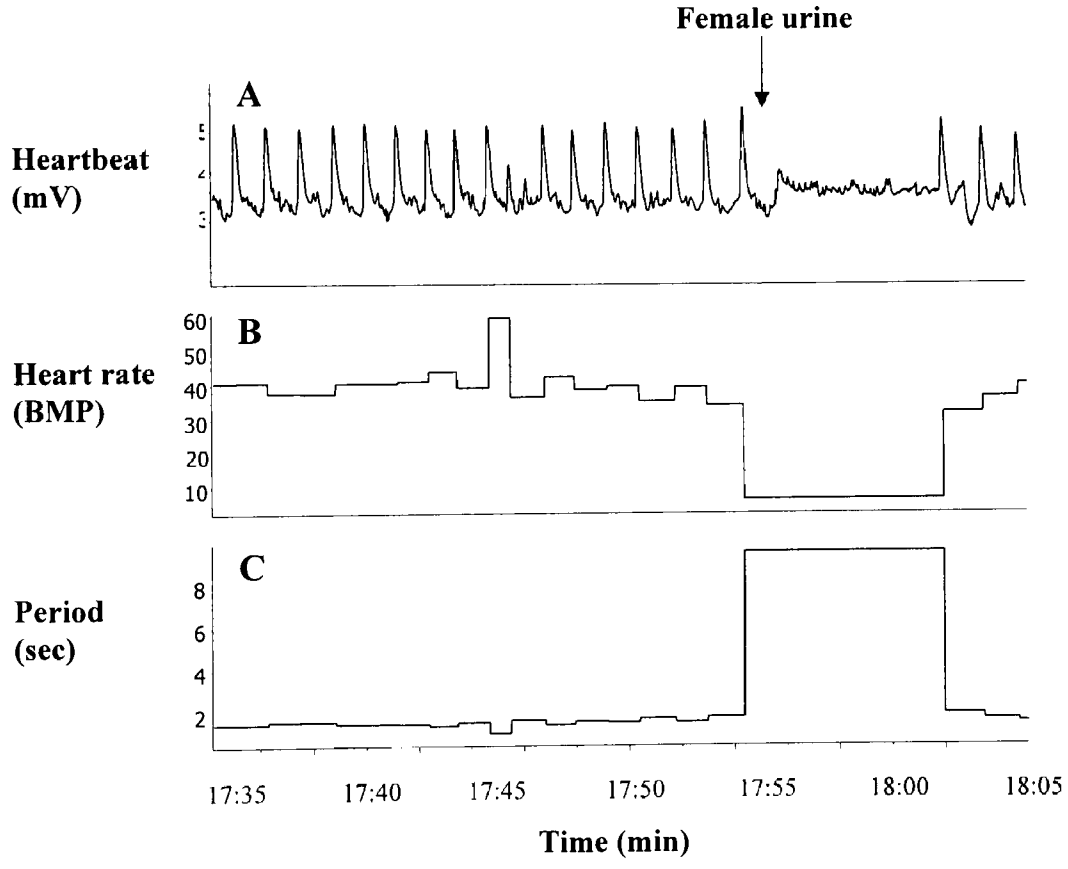

Figure 7.3: Example of a heart rate recording for a male crayfish following the release of female urine. (A) original heartbeat recording (B) instantaneous heart rate recording, beats per min (C) instantaneous recording of the interval between heartbeats (period) in seconds. The male displays a positive response to the female urine as the period of the heartbeat post stimulus is outside the confidence limit of the 20 pre-stimulus heartbeats.

Significantly more males showed cardiac arrest on the introduction of female urine in comparison to the water control (Fisher's exact test; $p < 0.05$ figure 7.4), with 75 % of males displaying cardiac arrest (defined as a post stimulus heartbeat period that was outside the confidence limits for the mean of the 20 pre-stimulus heartbeats, see figure

7.3). There was no difference in the number of positive responses between control water and male urine (Fisher's exact test; $p = 0.27$) or male urine and female urine (Fisher's exact test; $p = 0.27$).

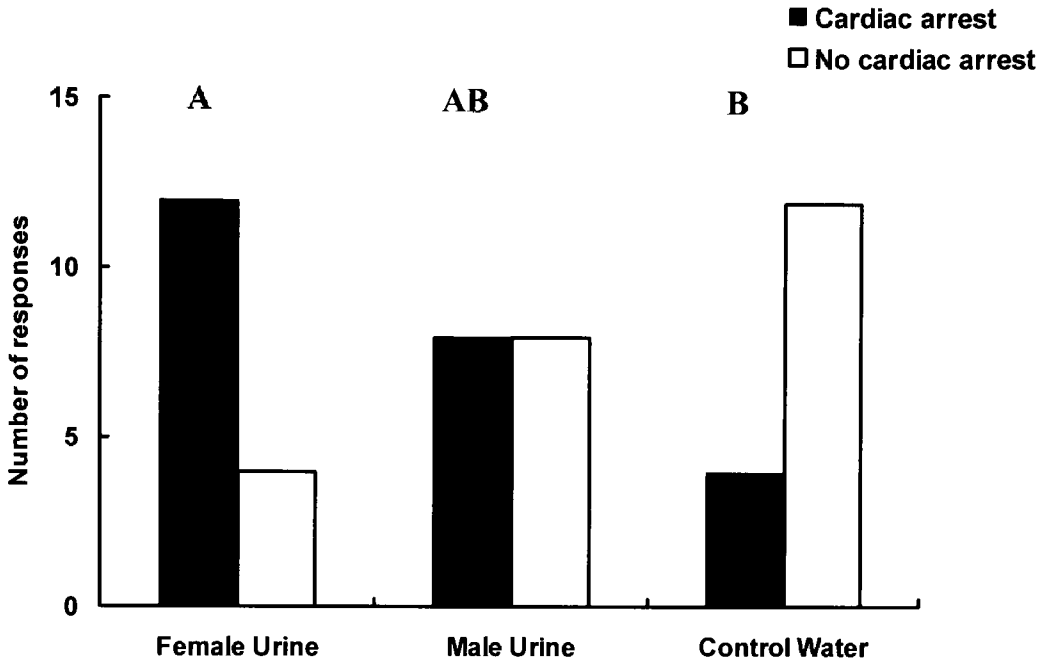

Figure 7.4: The number of male crayfish displaying cardiac arrest (black bars) or no response (white bars) to introduced stimulus. Significantly more males displayed cardiac arrest on introduction of female urine in comparison to the control water. Cardiac arrest was defined as a heartbeat period that was outside the confidence limits for the mean of the 20 pre-stimulus heartbeats. Values are total number of males responding ($N = 16$). Bars not connected by the same letter are significantly different ($p < 0.01$; Fisher's exact test).

Following the introduction of female urine there were changes in the instantaneous heart rate of male crayfish (figure 7.5; Friedman test; $\chi^2 = 20.625$, df = 3, $p < 0.001$). Immediately after stimulation, the heart rate declined from pre-stimulus levels (Wilcoxon Signed Rank; $p < 0.01$) as a result of male crayfish displaying cardiac arrest. Five min post-stimulation heart rate increased significantly from pre-stimulus levels (Wilcoxon Signed Rank; $p < 0.05$). Heart rate dropped back to pre-stimulus levels by 10 min post-introduction (Wilcoxon Signed Rank; $p < 0.05$). This pattern was not evident when male urine or control water was introduced (Friedman test; control water, $p = 0.42$; male urine, $p = 0.93$).

There was no significant difference between treatments in the standardised heart rate of males exposed to female urine, male urine or control water immediately after

stimulation (figure 7.5; Kruskal Wallis; p = 0.72), 5 min post-stimulation (Kruskal Wallis; p = 0.14) or 10 min post-stimulation (Kruskal Wallis; p = 0.69).

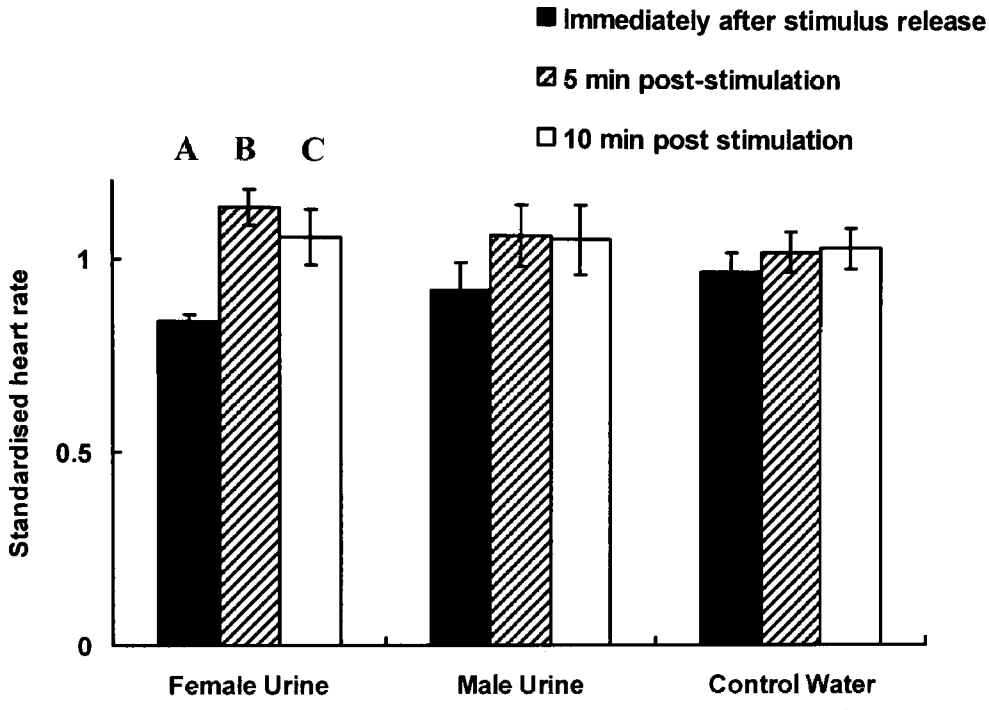

Figure 7.5: The standardised heart rate (post-stimulus heart rate/pre-stimulus heart rate) recorded by male crayfish (N = 48), immediately after stimulus release (black), 5 min post stimulation (striped) and 10 min post-stimulation (white). A score of one indicates no change between the mean pre-stimulus heart rate and the mean post stimulus heart rate. On introduction of female urine heart rate dropped as a result of male crayfish displaying cardiac arrest. Heart rate then increased significantly 5 min post stimulation before dropping to pre-stimulus levels at 10 min post stimulation (Bars not connected by the same letter are significantly different; $p < 0.05$ Wilcoxon signed rank post hoc tests). This pattern was not evident following stimulation with male urine or control water. There was no significant difference between standardised heart rate when comparing between treatments; immediately after stimulus release, 5 min post-stimulation or 10 min post stimulation. Values are mean ± S.E.

7.3.3 Ventilation assay

Prior to stimulation the scaphognathite beat period displayed by male crayfish ranged from 0.29-1.78 sec, post-stimulation the range was 0.28 - 3.73 sec. Upon stimulation male crayfish rarely displayed ventilatory arrest and there was no significant difference between the number of male crayfish displaying arrest upon introduction of female urine, male urine or the water control (figure 7.6; Chi-squared test; p = 0.49).

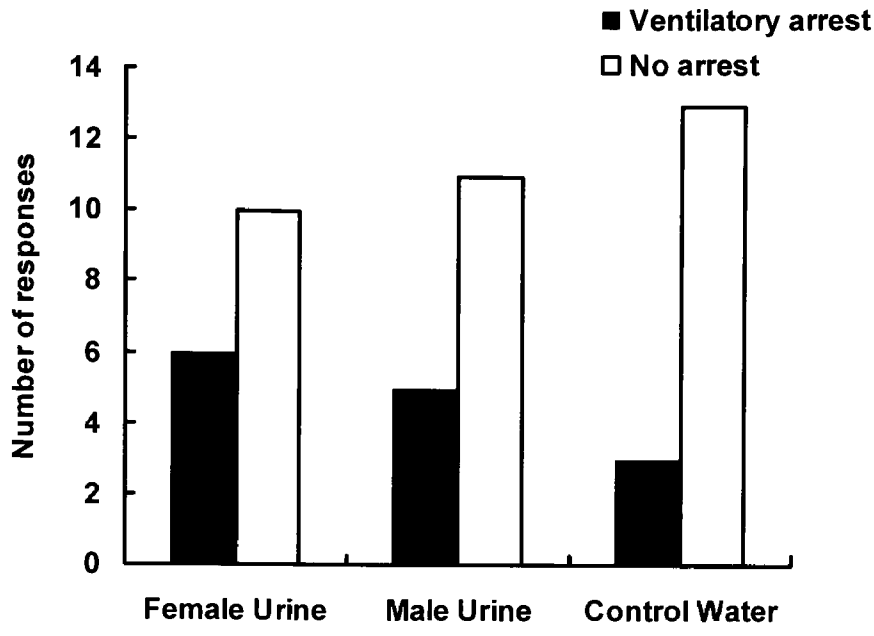

Figure 7.6: The number of male crayfish displaying ventilatory arrest (black bars) or no response (white bars) to introduced stimulus. There was no significant difference in the number of responses by male crayfish to stimulation by conspecific urine or control water. Ventilatory arrest was defined as a scaphognathite beat period that was outside the confidence limits for the mean of the 20 pre-stimulus scaphognathite beats. Values are total number of males responding (N = 16).

In addition, there was no significant difference in the standardised ventilation rate at either 0min, 5min, or 10min after stimulation with female urine (Freidman Test, p = 0.78), male urine (Friedman Test, p = 0.11) or control water (Freidman Test, p = 0.14).

7.4 Discussion

Previous studies on crayfish sex pheromones have yielded contradictory results on whether male crayfish can discriminate male and female odours (Ameyaw-Akumfi & Hazlett 1975; Dunham 1978; Itagaki & Thorp 1981; Thorp 1984; Stebbing et al. 2003a). This study indicates that male crayfish can distinguish conspecific male and female odours. We found specific behavioural as well as physiological responses in males to be more consistent when exposed to female urine or female conditioned water than to male urine, male conditioned water or control water. This supports the growing number of studies which indicate that urinary signals play a significant role in regulating social interactions of freshwater crayfish (Zulandt Schneider et al. 2001; Breithaupt & Eger 2002; Simon & Moore 2007; also see chapter three).

This study aimed to design an effective bioassay which would aid in the chemical identification of crayfish sex pheromones. Whilst we had success eliciting specific mounting behaviours and clear physiological responses, neither bioassay completely fulfilled both objectives of a quick and unambiguous test. The merits and weaknesses of the behavioural and physiological assays will be discussed below in relation to the complex nature of crayfish mating behaviour.

7.4.1 Behavioural bioassay

Male crayfish mounted the female dummy for significantly longer after the release of female urine in comparison to male odours and the water control. Mounting is an important part of pre-mating behaviour as it prepares the male for spermatophore deposition. Recent bioassay studies have delivered odour to the male subject through an airstone and although crayfish increased 'handling' behaviours in response to mature female odours, specific sexual behaviours could not be distinguished (Stebbing et al. 2003a; Belanger & Moore 2006). Despite releasing odours through a female dummy we could not elicit the full sequence of pre-mating behaviours including seizing (grabbing the rostrum), turning (turning the female so ventral surfaces are facing) and spermatophore deposition. Mounting may be regarded as unspecific since males may climb on objects for other reasons than mating. An example observed in our experiments was when males climbed on the dummy when trying to escape the tank (see methods). Mounting has also been observed in some cases of fighting behaviour between two male crayfish (*P. clarkii*), with the dominant male mounting the subordinate (Issa & Edwards 2006). Although this mounting behaviour would have had sexual origins it appears to have evolved into ritualised pseudocopulatory behaviour involved in the formation and maintenance of dominance relationships (Issa & Edwards 2006).

The lack of sexual behaviours, other than mounting, in our bioassay reflects the complexity of crayfish mating behaviour and the aquatic environment they inhabit. The success of studies on lepidopteron sex pheromones can be attributed to the simplicity of the animal's reaction to the pheromone, once a moth has entered the odour plume they fly directly to its source (Wyatt 2002). In crayfish there is no evidence for far field attraction of males by female pheromones. Alternatively individual crayfish may randomly come into contact and recognise sex during precopulatory aggression (Mason 1970; Villanelli & Gherardi 1998; Stebbing et al. 2003a) through physical, visual and

chemical signals (chapter three; Villanelli & Gherardi 1998; Acquistapace et al. 2002; Breithaupt & Eger 2002). Due to the complex nature of fight bouts and mating behaviours a bioassay which is based on the presentation of a single chemical stimulus might not be enough to elicit a full suite of mating behaviours. Courtship may consist of a chain of innate behaviours, with each stage consisting of a releasing stimulus and a specific response (Tinbergen 1951). Signal components that are presented by live females may be missing in our bioassay (e.g. hydrodynamic simuli), explaining the absence of turning and seizing behaviours.

Not all crustaceans require multiple releasing stimuli to elicit mating behaviours. Male shore crabs (*C. maenas*) presented with a stone or sponge treated with female odour will display the cradle carrying behaviours which are typical for courtship without the need for more specific visual or tactile cues (Hardege et al. 2002). This behavioural assay has recently been the sole guidance in the successful purification and identification of the shore crab sex pheromone (Bublitz 2007). Unfortunately crayfish are intermoult breeders and therefore lack the clear mate-guarding behaviour of soft shell maters, such as *C. maenas,* resulting in alternative behaviours such as mounting being key to bioassay development. Despite the lack of multimodal stimulation, males showed distinct responses to female odourants in our bioassay.

7.4.2 Physiological assays

Results from the heartbeat assay demonstrate that a rapid change in the heart rate of male crayfish can be induced through exposure to odour from conspecific male or female crayfish. The cardiac arrest response was very sensitive and allowed a quick and reliable assessment of whether the male had perceived the introduced stimulus. This response could be attributed to autonomic control of the cardiovascular system, commonly associated with 'fight or flight' responses described for vertebrates (Nicholls et al. 2001). Behavioural studies have shown crayfish are capable of displaying rapid defence reactions. Cardiac responses to chemical stimuli, including a short transient cardiac arrest followed by an increase in cardiac activity, have been recorded in this study (see figure 7.5) as well as in earlier studies of crayfish (Wiersma 1961; Schapker et al. 2002). This suggests that animals are primed in social situations in a similar way as in 'fight of flight' type responses. The ability of an individual to be alert to subtle changes in their environment may help coordination of intrinsic factors, such as oxygen

requirements, in preparation for imminent interactions, without the need for external behavioural changes (Li et al. 2000; Listerman et al. 2000; Schapker et al. 2002).

Previous studies have shown that ventilation rate in crayfish increases during aggressive social interactions (including prior to contact during visual displays) where active gill currents transport urinary signals to their opponents (chapter six; Breithaupt & Eger 2002; Schapker et al. 2002). In addition results from chapter five indicate that male crayfish can display ventilatory arrest at the onset of mating during seizing and turning behaviours, potentially as a result of reduced signalling by male crayfish (chapter three and five). However despite the biological function of gill currents in signalling behaviour the ventilation rate assay was not an accurate physiological indicator for male perception of introduced conspecific urine, when compared to the heartbeat assay. It is possible that when encountering the dummy male crayfish are missing vital hydrodynamic or mechanical stimuli which are essential perception of urinary signals. Using cardiac activity, rather than ventilatory activity, in a physiological assay may be more accurate as there is less intra-individual variation in pre-stimulus recordings than ventilation rate measures. This makes it easier to detect changes and repeat results from heartbeat assays in comparison to ventilation assays.

The use of the heartbeat assay could aid the chemical identification of crayfish sex pheromones by providing a very sensitive and quick assay to assess male crayfish responses to introduced odours. Physiological assays using heart rate could be employed in a similar manner to electro-olfactograms in fish studies (Stacey & Sorensen 2005), where assays indicate substances or fractions to which an individual is particularly sensitive. Analysis of the physiological responses is very quick allowing more replicates to be done than behavioural assays within the restricted breeding season of signal crayfish.

Although the heartbeat assay was very sensitive, the response of crayfish to conspecific odour was not sex-specific. Males reacted to both male and female odours through cardiac arrest. Male stimuli, however, were responded to in only 50 % of the experiments. This may be due to the stimulus originating from either a dominant or subordinate male. In the present study we did not control the dominance status of the donor animal providing the male stimulus. During agonistic bouts urine is released as a threat signal, predominately by the dominant animal, reducing aggression in the sub-

ordinate opponent (chapter three; Breithaupt & Eger 2002). Previous research has suggested that the aggressive state of crayfish is hormonally modulated by biogenic amines, such as serotonin, octopamine and dopamine (Edwards & Kravitz 1997; Huber et al. 1997a; Huber et al. 1997b). Intrinsic levels of these biogenic amines have been found to be elevated in the winner of contests between shore crabs (Sneddon et al. 2000). The metabolites of biogenic amines are excreted in the urine of crayfish and could potentially provide information on the intrinsic aggressive state of an individual to its opponent (Huber et al. 1997a). It is expected that dominance status has an influence on the potency of the urine stimuli and therefore the social status of the donor animal should be controlled during future bioassay procedures.

Ultimately, physiological tests should be complemented by behavioural assays which elicit courtship specific behaviours. This will ensure that identified chemical components mediate the specific biological function under study.

In conclusion, crayfish perform complex mating behaviours making it difficult to design and execute a bioassay based on specific sexual responses. However our study demonstrates how specific mounting behaviours can be elicited in male crayfish on stimulation with female urine. Although ventilation rate assays proved non-specific, physiological assays based on heart rate responses could be combined with the behavioural assay and should allow more replicates to be carried out within the restricted breeding season, driving fractionation and analytical purification of crayfish sex pheromones.

Chapter 8

The effects of artificial urine release on the fight dynamics of crayfish, *Pacifastacus leniusculus*

8.1 Introduction

Aggressive interactions are common in many animals where individual's need to resolve disputes over vital resources, such as food, space and sexual partners (Wilson 1975). The use of ritualised behaviours in the formation of dominance hierarchies can help weaker animals avoid injury whilst allowing dominant animals to avoid unnecessary energy expenditure and secure access to resources (Maynard Smith & Parker 1974; Parker 1976; Briffa & Sneddon 2006).

Dominance hierarchies can be maintained through two stabilising mechanisms; an assessment hierarchy or a confidence hierarchy (Barnard & Burk 1979). Confidence hierarchies are formed when previous agonistic experience influence an animal's fighting ability. Winners are more confident and subsequently win more fights and losers are more cautious resulting in more losses; the winner-loser effect (Barnard & Burk 1979; Chase et al. 1994; Hsu & Wolf 1999). By comparison, assessment hierarchies require animals to evaluate their opponent's agonistic potential against self abilities. Agonistic potential can be advertised through; visual cues (body size, weaponry), chemical cues (indication of internal physiology), or, mechanical cues (strength of attack). Individuals may assess the status of their opponent by recognising their individual identity (through individual recognition cues) or by assessing the dominance status of their opponent through status recognition cues.

Dominant-subordinate relationships for decapod crustaceans are normally established through agonistic interactions, where ritualised behaviours are displayed. For example, crayfish and lobsters initiate fights through approaches and threat displays, followed by escalation through a series of aggressive behaviours (claw push, box, grasp, tear) until one animal retreats or tail flips away (Huber & Kravitz 1995).

Dominance relationships between lobsters, *Homarus americanus*, are mediated through individual recognition cues, with second fights between familiar opponents having significantly reduced fight duration in comparison to initial fights (Karavanich & Atema 1998a). When paired with unfamiliar challengers, subordinate males fight to beat previously dominant opponents with repeated fights between unfamiliar opponents being of similar duration to initial fights (Karavanich & Atema 1998a). These results imply that dominance hierarchies are maintained by the loser's olfactory recognition of

the individual composition of the urine signature of the dominant male it had previously fought.

In contrast to lobsters, crayfish do not appear to recognise the individual identity of previously fought opponents. Crayfish form linear dominance hierarchies that are relatively stable with fight intensity and duration decreasing with time (Issa et al. 1999; Goessmann et al. 2000). Previous studies have shown that second fights between crayfish are shorter in duration than initial fights. However there is no significant difference in the duration of second fights between familiar and unfamiliar crayfish opponents (Zulandt Schneider et al. 2001; Breithaupt & Eger 2002). Crayfish therefore potentially recognise dominance status cues. In addition, dominance hierarchies may be reinforced by the winner loser effect, as the winner from previous fights may fight for longer and refuse to give up as easily due to increased confidence (Bergman et al. 2003; Hock & Huber 2006).

Dominance assessment between crayfish may be mediated by chemical signals released in the urine (Zulandt Schneider et al. 1999; Zulandt Schneider et al. 2001; Breithaupt & Eger 2002). The timing of urine release can be controlled, with animals increasing release during social interactions and often at times which will maximise the reception of signals by opponents (chapter three; Breithaupt & Eger 2002). The use of the dye fluorescein as a visual marker for urine release has demonstrated that crayfish release urine in association with aggressive behaviours (chapter three) and offensive behaviours are more effective when performed in conjunction with urine release (Breithaupt & Eger 2002).

Urine release and the reception of urinary cues have been indicated as playing key roles in the establishment and maintenance of crayfish dominance hierarchies (Zulandt Schneider et al. 2001; Horner et al. 2008). Prevention of chemical communication, through ablation of sensory organs appears to inhibit the formation of dominance hierarchies (Horner et al. 2008). Repeated fights between familiar red swamp crayfish opponents without chemosensory abilities were of similar duration and contained a similar number of fight bouts in comparison to initial fights (Horner et al. 2008). In addition, when urine release was blocked during *Orconectes rusticus* agonistic interactions, fights were longer and more intense than those where individuals could

release urinary signals to facilitate dominance recognition (Zulandt Schneider et al. 2001).

The chemical identity of urinary status cues in crustaceans is currently unknown. It also remains to be established whether a single component or a chemical mixture mediates dominance recognitions. Chemical identification of dominance odours would help to provide insights into the mechanisms of dominance recognition but also will indicate if these odours play an important role in female mate choice. Studies of the mating system of cockroaches (*Nauphoeta cinerea*) have indicated that females are capable of distinguishing male quality based on components of threat signals released in male-male competition (Moore 1997).

It can be hypothesised that the neurochemical differences between dominant and subordinate crayfish are expressed externally in the urine, communicating status. In order to identify any chemical components which distinguish dominant urinary cues from subordinate urinary cues a suitable bioassay needs to be developed to complement analytical chemistry techniques. Although considerable research effort has been directed towards designing a bioassay which can be used to identify crayfish sex pheromones (Stebbing et al. 2003a) there has been limited research into assays for dominance cue recognition. Stimulation of male crayfish to conspecific male urine has previously been observed as a test of sex specificity rather than to develop a specific test for male dominance cue identification. For example male crayfish (*Procambarus clarkii*) have been shown to respond to male odours by displaying aggressive postures (chelae raised) in comparison to submissive postures to female odour (chelae down) (Ameyaw-Akumfi & Hazlett 1975).

Previous work in this thesis (chapter seven) has indicated that physiological indicators (heart rate, ventilation rate) of male *Pacifastacus leniusculus* responses to stimulation by conspecific male odour may not be sensitive enough to form the basis of a suitable bioassay. Therefore a novel bioassay should be developed based on behavioural responses to stimulation. This assay needs to be able to distinguish differences between stimulation with dominant urine in comparison to subordinate urine.

This experiment aims to assess the role that chemical cues play during repeated fights between size matched crayfish (*P. leniusculus*). Opponents will fight twice, with urine

release unblocked on day one and blocked on day two. To assess the effectiveness of chemical communication urine will be reintroduced (from dominant or subordinate opponents) during a second day fight, when animals are performing aggressive behaviours. Results from these trials can then be compared to a control experiment where water is introduced during second day fights.

8.2 Materials and methods

8.2.1 Animals holding conditions

Adult male crayfish (*P. leniusculus*) were obtained in May 2008 from a crayfish dealer (Chris Campbell, North Dorset) having been trapped in a local lake. Animals were maintained in communal holding tanks (91.5 x 30 x 30 cm) at 17°C with a light cycle of 14:10h light: dark. A maximum of 25 animals were kept in each tank. Crayfish were fed twice weekly on commercially available defrosted prawns.

To study social interactions we used 90 intermoult male crayfish (mean ± SE carapace size of 35.7 ± 0.4 mm, mass 34.3 ± 1.1 g), all with intact appendages.

One week prior to fight encounters crayfish were isolated in separate 3 l PVC containers (24 x 17.5 x 8 cm), this should limit the effects of any prior social experiences (Zulandt Schneider et al. 2001; Hemsworth et al. 2007). To eliminate the effects of body size on fight outcome crayfish were size matched (carapace and chelae length differences less than 5 %). For identification, crayfish were individually numbered using white corrective fluid.

Twenty-four hours prior to fight one, crayfish were blindfolded by wrapping opaque material (1 cm x 4 cm) around the eyestalks and rostrum and securing excess material to the carapace using cyanoacrylate glue. Animals were blindfolded to prevent visual disturbances during stimulus introduction influencing behaviour in fight two.

8.2.2 Urine blocking

Crayfish pairs participated in two rounds of fighting. In fight one, nephropores were unblocked, so opponents could exchange chemical signals and establish a dominance hierarchy. In fight two nephropores were blocked, preventing urine release by both crayfish. To assess the affects of urinary signalling on fight dynamics control water, urine from the dominant male or urine from the subordinate male (as established in fight

one) were introduced during fight two. Ninety fights were performed in total, 45 on day one and 45 on day two (N = 15 per treatment).

Animals were treated using the same procedures prior to fight one and fight two. Three hours prior to each fight animals were strapped to a plastic board using elastic bands, so the ventral surface was exposed. One cm of silicon tubing (1.6mm diameter, Bio-Rad, USA) was attached to the basal segment of the second antenna, using cyanoacrylate glue (Zap-a-Gap, Pacer). To ensure the tubing was secure an additional cyanoacrylate layer was applied around the tube and dried using an accelerator fluid (Zip kicker, Pacer). Prior to fight one tubing was attached adjacent to the nephropore. For second day fights the tubing was secured around the nephropores.

Ten min before each fight plasticine was inserted into the tubing to act as a plug. The plasticine plug would not block urine release in fight one but provided a reversible urine block in fight two. Dye studies were conducted to ensure that the plasticine plug was efficient at blocking urine release from fighting crayfish. Fluorescein dye was used as a marker for urine release (for methods see Breithaupt & Eger 2002). The silicon tubing complete with plasticine plug was efficient at preventing urine release in all animals tested (N = 6).

Following each fight the silicon tubing and plasticine plugs were carefully removed. This form of urine blocking was reversible, with animals recovering quickly. No animals died as a result of this treatment.

8.2.3 General fight procedure

Fight one was performed 24 hours prior to fight two. Previous studies have indicated that under these time and fight conditions crayfish will have the energy reserves to produce similar fight duration and intensities (Zulandt Schneider et al. 2001; Breithaupt & Eger 2002).

Fights were carried out in a glass aquarium (30 x 20 x 20 cm) which was lit using a 60W desk lamp. Fights started after a 15 min acclimation period, where animals were physically separated by an opaque divider. Interactions were filmed (Panasonic digital video camera, NV-GS230) until dominance status had been established and for a minimum of 5 minutes after animals first came into contact. Dominant animals were

identified as those which initiated fights, showed high levels of aggression and did not show submissive behaviours (moving away from their opponent, fleeing using tail-flipping). Subordinate animals displayed comparatively low levels of aggression and following submissive behaviours did not re-engage in fights.

8.2.4 Stimulus collection and preparation

On completion of the first fight a urine sample was collected from both opponents. A micropipette tip (1-10 µl, Fisherbrand) was carefully inserted into the opening of the nephropore which caused urine to flow out from the bladder, to be collected by suction into Teflon tubing (1.5 mm diameter) and a 1.5 ml collection vial (method modified from Bamber & Naylor 1997). At least 200µl was collected from each individual. Urine samples were clearly labelled as dominant or subordinate and were frozen for 24 hrs at -25°C. Urine was collected from all animals (N = 90) which participated in the experiment to ensure crayfish were treated equally between fights one and two.

To ensure control water was kept under the same conditions as urine, water samples were also frozen 24 hr prior to second day fights. All water used in the experiment was filtered through a 25 cm pre-sediment filter followed by a 25 cm activated carbon filter (Pozzani Pure Water PLC, UK).

8.2.5 Stimulus introduction in fight two

Prior to the second fight a 250 µl syringe (Hamilton, Switzerland), fitted with a 5 cm needle (0.8 x 50mm, Microlance, Ireland) was filled with defrosted experimental stimulus. In each trial a single stimulus was introduced into the tank; either (a) urine from the dominant male (b) urine from the subordinate male, or, (c) control water.

Stimulus was introduced into the tank by placing the needle tip between the two crayfish at the level of their nephropores, this was done carefully to provide minimal disturbance of the water column. Stimulus was introduced in pulses when animals were in contact and fighting using their chela(e) to (i) push or box their opponent (ii) to grasp onto their opponents body (iii) in periods of unrestrained aggression. Pulses were introduced at times which reflected the natural urine release patterns between fighting male crayfish (as studied from experiments where fluorescein dye was used as a visual marker for urine release, chapter three). On completion of a urine pulse the needle was removed from the tank. To standardise introduction between treatments, stimulus was

introduced at the onset of agonistic behaviours, or, when animals increased their level of aggression. This encompasses times when animals do not change behavioural category but increase their aggression within a behavioural category, which often occurs after a pause. A maximum of 200µl was used in any one trial.

8.2.6 Behavioural analysis

Filmed fights were digitised and then analysed using a behavioural software package (The Observer 5.0, Noldus Information Technology, Wageningen, The Netherlands) which allowed behaviours to be scored on a continuous time-scale. Each trial was assigned a code so the analyser did not know the identity of the released stimulus in fight two.

Initial fight analysis indicated that signal crayfish established dominance relationships in fight one within 5 min after first contact. Therefore trials were only analysed for the 5 min after initial contact. Experiments where the dominance hierarchy had not been established prior to 5 min were discounted from analysis (N = 3).

Total fight duration was determined by timing when animals were engaged in physical contact over the 5 min experimental period. This incorporated times where animals initiated fights, used claws to push and box opponents, used the claws to clamp onto the opponent's body or chela(e) or when animals used unrestrained levels of aggression. Fight bouts ended when one animal (the loser) moved away from the opponent and one body length separated them. Within one trial there could be multiple fight bouts. The total fight duration for each trial was the summed duration of all bouts.

For each trial the subordinate animal would determine when a fight bout was over, by moving or backing away from the opponent. Therefore to measure whether the intensity of fights changed between fight one and two an aggression score was assigned to the subordinate animal. The score was calculated by measuring the proportion of time the subordinate animal spent displaying highly aggressive behaviours (claws clamping onto opponents body, unrestrained levels of aggression) in relation to the total amount of time spent engaged in physical contact.

8.2.7 Statistical analysis

Prior to analysis all data was tested for normality (Kolmogorov-Smirnov test) and homogeneity (Levene's test); tests showed data was normally distributed with equal variances between groups, therefore parametric tests were used. Separate two-way repeated measures ANOVAs were used to investigate differences in fight duration and fight intensity between treatments (between subjects factor: control water, dominant urine, subordinate urine) and fight encounter (within subjects factor: fight one vs fight two). Where a significant interaction between treatment and fight encounter was found post hoc analysis was performed using a Tukey's HSD test.

8.3 Results

There were significant differences in the dynamics of fights between crayfish with unblocked nephropores on day one and blocked nephropores on day two; dependant on whether they were exposed to control water, subordinate urine or dominant urine during the second day fight (figure 8.1; table 8.1; fight duration; two-way repeated measures ANOVA; $F_{2,42} = 3.45$, $p < 0.05$: fight intensity; two-way repeated measures ANOVA; $F_{2,42} = 3.53$, $p < 0.05$). When animals were exposed to dominant urine on day two, second fights were significantly shorter (Tukey's HSD post hoc test; $p < 0.05$) than initial fights and subordinates showed significantly lower fight intensity (Tukey's HSD post hoc test; $p < 0.05$) than on the first day. In comparison, there was no difference in the duration or aggression score of encounters between animals exposed to control water or subordinate urine on day two (Tukey's HSD post hoc test; $p > 0.05$).

Table 8.1: Two-way repeated measures ANOVA results for (A) fight intensity and (B) fight duration of second day fights where crayfish had blocked nephropores and were exposed to different experimental treatments (dominant urine, subordinate urine, control water). Within subject factor – day of fight; between subject factor – treatment).

A

Factor	df	F	P
Treatment	2	2.18	0.13
Day of fight	1	16.72	< 0.001
Interaction	2	3.53	0.04
Total	42		

B

Factor	df	F	P
Treatment	2	3.53	0.04
Day of fight	1	3.95	0.53
Interaction	2	3.45	0.04
Total	42		

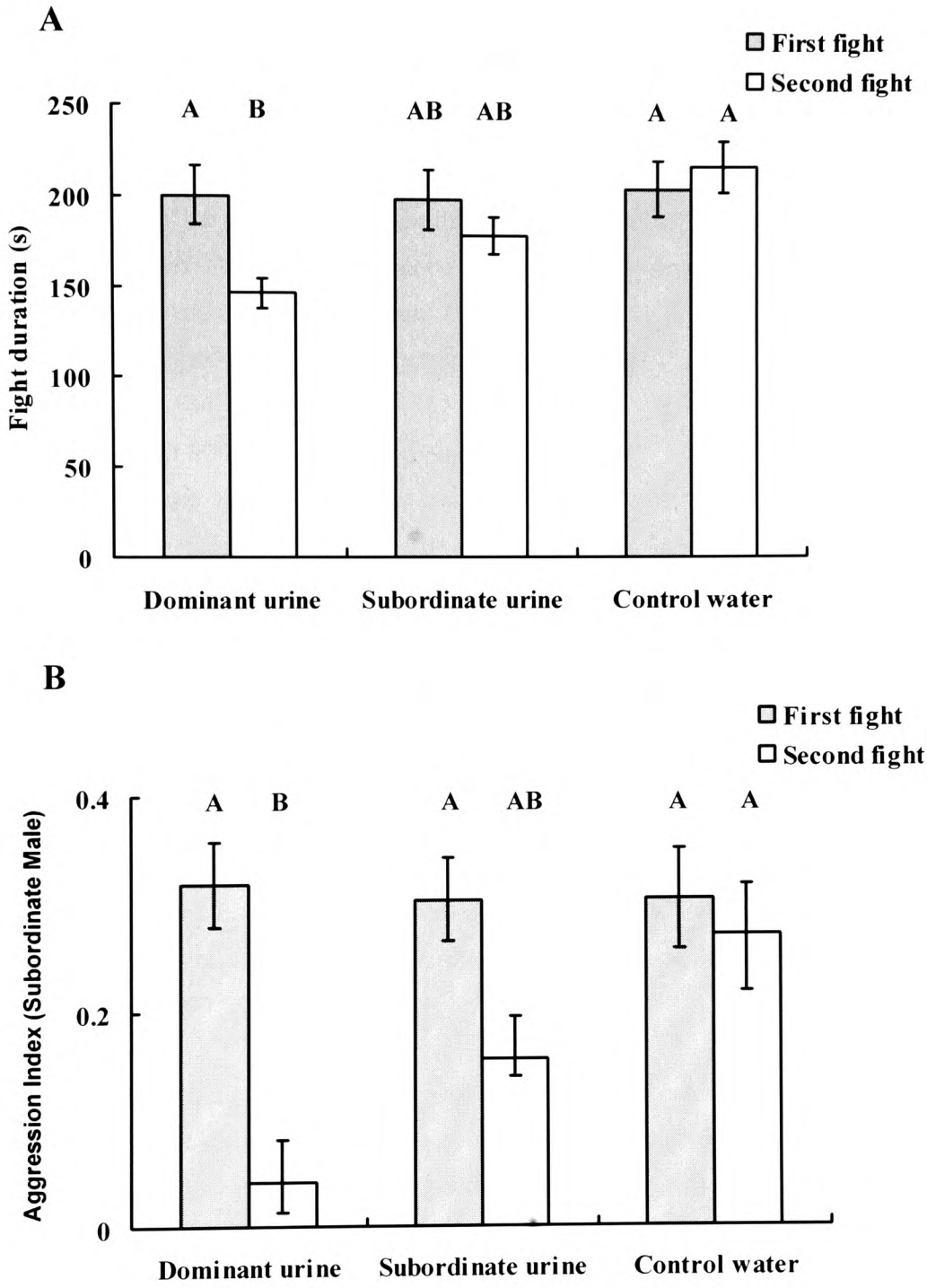

Figure 8.1: The mean (A) fight duration and (B) fight intensity of crayfish with unblocked nephropores on day one (grey bars) and blocked nephropores on day two (white bars). During the second day fight crayfish pairs were exposed to dominant urine, subordinate urine or control water. N = 15 pairs for each treatment. Fight intensity is represented by the aggression index representing the proportion of total interaction time the subordinate male spent engaged grasping the opponent or combating using unrestrained aggression. Fight duration and intensity was significantly reduced on day two following the addition of dominant urine (Two-way repeated measures ANOVAs; $p < 0.05$). Bars not connected by the same letter are significantly different; $p < 0.05$, Tukey's HSD post-hoc test.

8.4 Discussion

Urinary signals have been shown to play a major role in mediating crayfish agonistic interaction and stabilising dominance hierarchies (chapter three; Zulandt Schneider et al. 2001; Bergman et al. 2003; Horner et al. 2008). This study provides the first direct evidence that it is the urine from the dominant male crayfish that causes the loser of the initial fight to give up early in the second fight and display lower levels of intensity. Therefore dominance recognition can be established by artificially introducing dominant urine signals, but not by subordinate urine or water.

8.4.1 The effect of dominant urinary signals in fights

This study indicates that it is the presence of urine from the dominant male which is the integral factor in influencing the duration of repeated fight bouts and the stability of dominance hierarchies. It appears that subordinate males will only retreat from a fight if they have a chemical signal from which they can assess the status of their opponent, potentially by comparing to self abilities. Our study highlights that chemical cues are more important in hierarchy formation than visual cues, which were absent in this experiment. These results support previous work which has shown that dominance relationships break down in the absence of urinary signals. Blocking urine release results in longer and more intense fights in comparison to when animals can communicate their status (Zulandt Schneider et al. 2001) and inhibiting chemosensory abilities can prevent individuals recognising the dominance status of their opponent (Bergman et al. 2003; Horner et al. 2008).

In addition to the assessment of urinary status cues, crayfish dominance hierarchies may be stabilised by the winner-loser effect (Copp 1986; Bergman et al. 2003; Hock & Huber 2006), where winners of fights are more likely to win future encounters than losers. The results of this study question the importance of confidence in the formation of hierarchies by *P. leniusculus*, as in the absence of any urinary signals second day fights were of equal intensity and duration as first day fights. In addition, in two control trials (out of 15) the subordinate opponent from initial fights out-competed their opponent on day two. It is possible that the influence of the winner-loser effect was lost over the 24 hr delay between fight one and fight two. Rather than being the principal mechanism mediating the formation of dominance hierarchies in crayfish it is more likely that animals assess their opponents using urinary cues, with the winner-loser effect potentially reinforcing the decision to fight or withdraw.

8.4.2 Candidate substances for crayfish status cues

The chemical identity of urinary status cues in crustaceans is currently unknown. Previous research has indicated that biogenic amines may play an important part in mediating agonistic interactions and regulating dominance hierarchies (Sneddon et al. 2000; Briffa & Elwood 2007; Tricarico & Gherardi 2007). In crayfish biogenic amines are important neuromodulators and neurotransmitters in the nervous system (Vogt 2002). Changes in internal haemolymph concentrations of octopamine, dopamine and serotonin are associated with differences in dominance status in shore crabs (*Carcinus maenas*) (Sneddon et al. 2000). Resting and post-fight levels of octopamine, dopamine and serotonin were all higher in winners in comparison to losers (Sneddon et al. 2000). Treatment of crayfish with exogenous injections of biogenic amines can alter postures, levels of aggression and the decision to retreat in fights (Livingstone et al. 1980; Huber et al. 1997b; Tricarico & Gherardi 2007). For example treating crayfish with octopamine results in displays of submissive postures and reduced aggression, whereas serotonin treatment induces displays of dominant postures and increased aggression (Livingstone et al. 1980; Tricarico & Gherardi 2007).

Biogenic amines are converted into sulphate conjugates in the tissues of lobsters as a way of inactivation. It is these metabolic products which are released in the urine rather than the amines themselves (Kennedy 1978). Formation of the sulphate bond in the conversion of serotonin requires 2 ATPs and serotonin-o-sulphate is therefore deemed an energetically expensive metabolite to synthesise (Huber et al. 1997a). This raises the possibility that its excretion in crustaceans is for signalling purposes rather than solely as a waste product. Therefore initial studies should focus on identifying the sulphate conjugates of biogenic amines released in the urine and any potential difference in their concentration, between dominant and subordinate opponents.

The methodology developed in the current study provides a promising bioassay to test the chemical nature of dominance status cues. Bioassay driven purification techniques combined with HPLC-MS should help to narrow the search for the active chemical components of crayfish urine. Identification of the dominance status cues may open new quantitative ways of studying crayfish behaviour, neurobiology and evolution. Behavioural studies could be performed to assess whether female crayfish are attracted to dominant status cues during the breeding season. Further chemical investigations

could also show whether the active chemical components of dominance cues are the same between different species of crayfish.

Chapter 9

Discussion

Pacifastacus leniusculus and *Procambarus clarkii* are highly adaptable species of crayfish and have become established pests throughout Europe, causing the decline of native species and the degradation of indigenous habitats (Holdich 1999; Souty-Grosset et al. 2006). It is therefore important to have comprehensive knowledge on crayfish behaviour in order to plan effective control and eradication programmes for nuisance populations.

This thesis focused on using behavioural and physiological techniques to investigate how crayfish (*P. leniusculus* and *P. clarkii*) utilise chemical signals in agonistic and reproductive behaviours (see chapters three – six). The results of these studies helped to focus the design of novel assays (see chapters seven/eight), which can be used to drive the purification techniques required to identify the active chemical components of crayfish sex pheromones and dominance pheromones. Identification of crayfish pheromones will open doors to new research areas and will help to assess the viability of the employment of pheromone baits in trapping regimes for the control of problematic populations.

9.1 Chemical communication in crayfish

Urinary signals were found to play a key role in regulating the social behaviours of crayfish, particularly during agonistic and reproductive behaviours.

9.1.1 Chemical signalling during mating

Ameyaw-Akumfi and Hazlett first reported the importance of crayfish sex pheromones in mate recognition and reproductive behaviours in 1975. However subsequent studies have debated the relevance of female sex pheromones, with several studies yielding contradictory results (Itagaki & Thorp 1981; Rose 1982; Bechler 1995; Stebbing et al. 2003a). This thesis shows that female chemical signals are integral in male recognition of partners and the initiation of reproductive behaviours. Male crayfish (*P. leniusculus*) respond to urinary signals from receptive females through both behavioural and physiological responses (see chapter three and seven). On reception of female urine, male crayfish decrease their level of aggressive signalling and initiate mating behaviours by seizing and turning the female (chapter three). Physiological responses were found to be brief cardiac arrest followed by an increase in heart rate (chapter seven). Similar responses were recorded for ventilatory activity during real time reproductive behaviours (chapter five) but were not reproduced when female odours

were introduced exogenously (chapter seven). These physiological responses will help to prime the male's circulatory system with oxygen prior to the physical exertion of mating.

Female urinary signalling has been shown to be integral to instigating male reproductive responses (chapter three). However results also indicate that female crayfish release urine as a threat signal, potentially to resist male mating attempts. It is likely that female urinary signals have not evolved as attractive courtship signals and instead male crayfish detect female receptivity by spying on hormones and metabolites released with urinary threat signals. This can be compared to the sex recognition system of multiple fish species, where hormonal cues influence conspecific behaviour and physiology (Sorensen & Stacey 2004; Stacey & Sorensen 2005). For example, female goldfish (*Carassius auratus*) release unmodified hormones in the urine, which induce strong behavioural and endocrinological responses in males. Pheromones have been fully categorised and females have been found to release both precopulatory steroid pheromones (which have multiple chemical components, including sulphate conjugates) and post-ovulatory prostaglandin pheromones ($PGF_{2\alpha}$ and metabolites) in urinary pulses (Stacey & Sorensen 2004). These signals advertise female readiness to spawn (Sorensen & Stacey 2004; Stacey & Sorensen 2005). By comparison, in many species of moths the females release highly specialised chemical courtship signals, which elicit male searching and release mating behaviours (Phelan 1997; Alexander et al. 1997). Here, both signal and response are adaptive and only minute traces of pheromone can be received by specialised male receptors to evoke sexual responses (Greenfield 1981).

Receptive female goldfish release urine more frequently when in the presence of a male, urinating into spawning substrates (Applet & Sorensen 2007). This suggests females are using urine as an attractive lure. By comparison urinary hormonal cues of female *P. leniusculus* are released in association with threat signals and aggressive behaviours (chapter three). During pre-copulatory aggression female crayfish miscommunicate their aggressive motivation as the release of threat signals elicits reproductive behaviours rather than defensive behaviours in the male (chapter three). This complicated urinary signalling system can explain the slow progress in designing a reliable bioassay for the identification of female sex pheromones. The combination of physical contact with urinary signalling may be required to elicit specific sexual responses in male receivers (seizing, turning, spermatophore deposition).

Investigations of the timing of urine release by other species of crayfish will indicate whether all crayfish display their receptivity through aggressive signalling. It would be interesting to draw comparisons to crayfish species which have a protracted breeding season, rather than the short season of *P. leniusculus*.

9.1.2 Aggressive chemical signalling

The formation of dominance hierarchies is extremely important in order to prevent excessive energy expenditure and risk of injury (Parker 1974; Maynard Smith & Parker 1976). The results of this thesis confirm that urinary signals mediate agonistic interactions between crayfish (chapter three), with aggressive signalling increasing the effectiveness of offensive behaviours (Breithaupt & Eger, 2002). Blocking urine release and artificially introducing urine samples (see chapter eight) showed that the loser of a fight is highly influenced by urinary signals from the dominant opponent. It is these threat signals which causes the loser to reduce displays of highly aggressive behaviours and give up earlier during second day fights. The methodology of blocking urine release and 'replaying' urine release provides a strong assay for driving future research on the chemical identity of dominance pheromones. This assay is based on unambiguous behaviours and is particularly promising due to its simplicity, reliability and speed, allowing multiple experiments to be performed over a short timescale. In this respect this assay shows far more potential than the sex pheromone assay developed in chapter seven, which requires the combination of both behavioural and physiological methodologies.

It can be hypothesised that differences in the internal motivational and physiological state of crayfish is communicated externally in urinary threat signals and it is this information that the subordinate animal is using to make decisions on when to give up in a fight. Biogenic amines have been proposed as dominance cues in a range of aquatic crustaceans (Huber et al 1997a; Sneddon et al 2000). Previous studies have linked differences in haemolymph levels of biogenic amines to fighting ability of shore crabs (*Carcinus maenas*) (Sneddon et al. 2000). Dominant crabs had higher haemolymph concentrations of octopamine, dopamine and serotonin, in comparison to losers. To date no study has linked differences in the concentration of biogenic amines in the urine with an individual's ability to win a fight. Do dominant crayfish have higher levels of biogenic amines in their aggressive urinary signals than subordinates?

9.1.3 Signal delivery

To fully understand how chemical signals are employed in the regulation of social behaviours it is important to know how signals are transported from the sender to the receiver. Self generated currents are important in aquatic environments, where molecular diffusion is extremely slow (Atema 1995). In a range of aquatic crustaceans, gill currents are produced during agonistic encounters, potentially to direct urinary signals towards the opponent and/or to provide specific hydrodynamic signals; e.g. the hermit crab *Calcinus tibicen* (Barron & Hazlett 1989), the velvet swimming crab, *Necora puber* (Smith & Talyor 1993) and the snapping shrimp, *Alpheus heterochaelis* (Herberholz & Schmitz 2001). Visualisation of crayfish urinary signals has shown that crayfish create gill currents through movements of the scaphognathites (paddle like appendages of the second maxillae) which can be used to distribute urinary signals during social interactions (Breithaupt & Eger 2002; Bergman et al. 2005). The direct correlation between increased ventilatory activity and the visualisation of urine release observed in chapter six also shows that crayfish use gill currents to disperse signals. The active use of gill currents will improve signal transfer efficiency from sender to receiver.

9.2 Future research

This thesis confirms the importance of crayfish chemical signalling during both reproductive and agonistic behaviours, which combined with the development of novel assay techniques, opens up several potential research areas:

9.2.1 Determination of the chemical nature of crayfish pheromones

To date only two crustacean sex pheromones have been chemically characterised (Asai et al. 2000; Bublitz 2007), with slow progression being attributed to poor bioassay design, often based on ambiguous sexual behaviours (Dunham 1978; Bechler 1995; Hardege et al. 2002). This thesis has developed two assays which can be coupled with analytical chemistry techniques (such as HPLC-MS) and used in the future to determine the active chemical components of crayfish sex pheromones (see chapter seven) and crayfish dominance pheromones (see chapter eight).

9.2.1.1 *Sex pheromones*

There is currently no candidate substance for the active chemical component of the female sex pheromone of signal crayfish. Future studies should therefore use the

physiological and behavioural assay developed here (see chapter seven) to check the biological activity of female urine following fractionation procedures. Unlocking the chemical identity of the female sex pheromone will help answer several key evolutionary, biological and neurological questions. For example; are sex pheromones produced by *P. leniusculus* species-specific and do they play a key role in the reproductive isolation of closely related species?

In this thesis it has been suggested that male crayfish spy on female hormones and metabolites released in the urine with threat signals. Hormone systems are highly conserved and therefore species-specificity of mate recognition may not arise, (with potential exceptions for sympatric species). In fish mating systems, which rely on the male detection of female hormones, there is a close correlation between phylogeny and the pattern of compounds detected (Sorensen & Stacey 2004). The pattern of steroid detection varies among cyprinid subfamilies but is similar within subfamilies (Sorensen 1992; Stacey & Sorensen 2005). This suggests that the evolution of hormonal pheromones has been constrained. There is, however, some potential for specificity, which may be achieved by altering ratios of the odour components or by using other contextual cues. It may therefore be expected that the sex pheromone of *P. leniusculus* may be effective at eliciting sexual responses in closely related species. By comparison moths use highly specialised, species-specific pheromones signals, where specificity can be achieved by multi-component blends of several pheromone components (Christensen et al. 1989).

Determining the chemical nature of crayfish signals would also help to shed light onto the maximum distance that male crayfish detect female pheromones. Male crayfish were found to recognise female mates during aggressive behaviours, it therefore may be expected that for *P. leniusculus* pheromones are only effective when animals are in close-contact, rather than working as a distance pheromone. The identified pheromone of the shore crab (*Carcinus maenas*), uridine-di-phosphate, has been shown to be attractive over 2 m, and elicits reproductive behaviours when animals are in close range (Fletcher 2007). Future experiments should test the effective range of female urinary signals. Can males detect females from a near, mid or far-field range? If female pheromones were found to be ineffective as a long range attractant this would call into question the application of pheromone baits for trapping invasive crayfish populations. It would be interesting to investigate whether ecological factors, such as habitat type

influences the range at which crayfish can detect chemical signals i.e. a comparison between species living in lentic or lotic water bodies.

9.2.1.2 *Dominance pheromones*

Biogenic amines and their sulphate conjugates (such as serotonin-O-sulphate) have been suggested as the active component of dominance pheromones (Huber et al. 1997a) and studies can therefore focus on identifying these components in crayfish urine. The urine blocking and artificial introduction assay designed in chapter eight can be used to assess if exogenous introduction of biogenic amines has the same effect on subordinate crayfish as urine from a dominant opponent. This behavioural assay can be coupled with analytical chemistry (e.g. HPLC-MS) to measure the relative concentration of amines in the urine of dominant versus subordinate crayfish. Discovery of crayfish dominance pheromones would answer questions about how dominance hierarchies are established and maintained. In addition, choice tests could be performed to assess if female crayfish show a preference for identified dominance cues.

The urine blocking assay developed in chapter eight could also provide a novel way to investigate the dominance pheromones in other decapod crustaceans, where urine release can easily be blocked and collected. 'Replaying' the urine from previously fought opponents proved an efficient and reliable assay for identifying the potency of dominant urine. Future studies could assess whether introduction of urine from unfamiliar donor crayfish (of known dominance status) is as effective at influencing a subordinate's decision to retreat and display low aggression behaviours. If this could be achieved this assay provides a really promising method for use in dominance pheromone identification of crayfish, and other decapod species.

9.2.2 Application of methodologies

Several methodologies have been developed throughout this thesis which can be used in future studies of the chemical communication of crayfish and other aquatic crustacean species.

The use of fluorescein as a visual marker for urine release has provided an important tool for investigating crayfish chemical signalling during agonistic and reproductive behaviours (Breithaupt & Eger 2002; chapter three). The application of this technique could be extended to other species of aquatic crustaceans. It would be interesting to

compare urinary signalling in crayfish, which are intermoult maters, with the signalling behaviour of a crustacean species where mating is tied to the moult cycle (such as *Carcinus maenas*).

Visualisation of urine can also provide an important tool for ensuring that blocking of urine release is effective (see chapter eight). The use of tubing to temporarily block urine release allows animals to perform natural behaviours (when comparing to blocking using a catheter; see Breithaupt et al. 1999) and can be used to assess the effects of urinary signalling on behaviours. Future studies could block urine release of receptive crayfish to answer the key question; can male crayfish recognise a receptive female partner in the absence of female urinary signalling? This should help to determine the relative importance of chemical signalling in comparison to visual, mechanical and other hydrodynamic signals for initiation and completion of mating behaviours.

Methods to measure changes in the heart rate and ventilation rate of adult crayfish were developed in chapter five and six. These non-invasive methodologies (adapted from Depledge & Andersen 1990) can be used to measure physiological parameters during a range of social behaviours and can be extended to other species of aquatic crustaceans. The results from chapter six showed that chemical signalling behaviour was closely associated with scaphognathite movements and associated gill currents. By measuring ventilatory activity it can be assessed whether other crayfish species (including examples from the astacid family and the parastacoidea) use gill currents to disperse chemical signals. Comparisons could be drawn between species living in lotic and lentic habitats; are gill currents more important for species living in habitats with minimal natural flow?

9.2.3 Mate choice studies

This thesis has revealed the importance of female pheromones during reproductive behaviours; however the importance of male pheromones is unresolved. Male crayfish were found to reduce chemical signalling during sexual interactions (chapter three) and mate choice experiments (chapter four) indicate that females can not distinguish between dominant and subordinate males through chemical signals alone. This leaves unanswered questions as to how female crayfish recognise the quality of potential partners and choose the best available male. This is especially important for female

crayfish when considering the high level of investment they invest in rearing their offspring (Reynolds 2002).

Future studies need to focus on deciphering the possible mechanisms females use to choose partners. Do females judge male quality on the basis of multimodal signals, incorporating chemical, visual and mechanical stimuli? Is a cryptic post-copulatory choice more important than a pre-mate choice, as may be expected in a system where sexual coercion is commonplace? Recent studies have shown that females are capable of adjusting egg and clutch size in relation to male traits, such as size (Galeotti et al. 2006). Future experiments can focus on controlling the partners of female crayfish and using paternity analysis to assess whether female actively select dominant male partners over subordinates using a post mate choice.

9.3 Conclusion

Crayfish perform complex reproductive and agonistic behaviours which have made it problematic to design reliable assays based on specific, biologically relevant behaviours. In particular, the finding that female urinary cues are not released as courtship signals and the lack of male advertisement signals may have caused difficulties in designing bioassays in the past. This thesis combines both behavioural and physiological studies to determine the effects of urinary signalling during crayfish social interactions. Two novel assays have been designed which will help to drive fractionation and analytical purification of crayfish sex and dominance urinary signals. Pheromone identification will help progress our understanding of crayfish ecological and evolutionary relationships, the neurobiology of signal pathways and the costs of signal production and delivery.

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