AN INTERDISCIPLINARY INVESTIGATION OF THE ROLE OF CRAYFISH MAJOR CHELAE IN THE DISCRIMINATION OF CONSPECIFIC ODOURS: FROM MORPHOLOGY TO BEHAVIOUR

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A Dissertation

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

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ABSTRACT

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This dissertation examines the use of the major chelae by male crayfish (*Orconectes rusticus*) for discrimination and localization of female odours and links this behaviour with sensory setae morphology and distribution. First, a behavioural bioassay was implemented to test whether male reproductive (form I) and non-reproductive (form II) crayfish use their major chelae to detect and discriminate reproductive female odours. Behavioural reactions of male crayfish, with intact or sensory blocked chelae, were recorded to four different odour treatments: female-conditioned water, male-conditioned water, fish homogenate, and water (control). Intact form I males handled the female odour source significantly more (46.4 ± 17.0 seconds) than blocked and form II males. Next, the role of sensory information from the major chelae in the localization of reproductive female odours was examined. Behavioural reactions of intact and blocked form I male crayfish were analyzed in response to three different odour treatments: female-conditioned water, male-conditioned water, and water (control) delivered from one end of a test arena. Large-scale movements and chemosensory sampling behaviours were measured. Male crayfish with intact major chelae spent more time (45.4 ± 7.7 %) closer to the odour source when the reproductive female odour was delivered compared to all other odours. Also, intact male crayfish spent more time chelae waving (61 %) in response to female odours than blocked males. Lastly, a comparative and morphological analysis of setae found on the major chelae of form I and II male crayfish was performed. To accomplish this, scanning electron microscopy, a permeability assay, anterograde labelling, and acetylated tubulin (AT) immunocytochemistry were used. Results show that form I crayfish had more sensory setae pockets and individual smooth setae on their major chelae compared to form II males. Also, smooth setae contained a
terminal pore, were crystal violet permeable, and contained anterogradely labelled DiI and AT positive nerve fibers. Conversely, plumose setae lacked a terminal pore, did not label with DiI, and were crystal violet and AT negative. Overall, these results suggest that the major chelae are important sensory structures needed for the localization and discrimination of female odours and may be important for reproductive behaviours such as mate recognition.
“Scientific knowledge is a body of statements of varying degrees of certainty—some most unsure, some nearly sure, but none absolutely certain.” Richard P. Feynman
To all of those who have **ALWAYS** been there for me throughout my Ph.D. with love, support, and encouragement.
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Paul A. Moore, as well as my committee members: Dr. Barbara Zielinski (University of Windsor), Dr. Vern Bingman, Dr. Tami Steveson, and Dr. M. Sue Houston for their advice and critique of this research. I would like to thank Dr. Zielinski for hosting me in her laboratory during 2004-2005 and providing much needed guidance and support for the morphology section of my dissertation. This research would not have been possible without help, support, and encouragement from lab mates and friends, past and present, including those in the Laboratory for Sensory Ecology at Bowling Green State University and the Zielinski Lab at the University of Windsor. Specifically, I would like to thank my lab mates for help in crayfish collection, aid in experimental design and implementation, and for the many hours they spent critiquing my manuscripts, seminars, and grant applications. Several thanks to the support staff at both Bowling Green State University and the University of Windsor. Also, Drs. Marilyn Cayer (Bowling Green State University), Michael Crawford (University of Windsor), and Margaret Yacobucci (Bowling Green State University) all provided equipment guidance and use of the microscopes housed in their laboratories. Funding for this research came from several sources including: Grants-in-Aid of research awarded to RMB from Sigma Xi and the Society for Integrative and Comparative Biology, fellowships awarded to RMB from both the J.P. Scott Center for Neuroscience, Mind & Behavior and the Department of Biological Sciences, and a National Science Foundation grant to PAM (IBN# 0131320). During my tenure at Bowling Green State University, other awards assisted in providing support for my research, these included: the Oman Graduate Scholarship, Katzner and University Bookstore Award, and the Charles E. Shanklin Award for Academic Excellence. Lastly, I would also to thank Bowling Green State University for five years of tuition waivers and teaching assistantships.
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<td>®</td>
<td>Registered</td>
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<td>I</td>
<td>Form I (reproductive)</td>
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<td>II</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AT</td>
<td>Acetylated tubulin</td>
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<tr>
<td>AT+</td>
<td>Acetylated tubulin positive</td>
</tr>
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<td>AT-</td>
<td>Acetylated tubulin negative</td>
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<td>BFIF</td>
<td>Blocked form I males to food odour</td>
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<tr>
<td>BFIIF</td>
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<td>CL</td>
<td>Chelae length (cm)</td>
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<td>IFIIIF</td>
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<tr>
<td>IFIFe</td>
<td>Intact form I males to female odour</td>
</tr>
<tr>
<td>p</td>
<td>Plumose setae</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>s</td>
<td>Smooth setae</td>
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<tr>
<td>S.E.</td>
<td>Standard error of the mean</td>
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<td>sec</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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CHAPTER I.

GENERAL INTRODUCTION

*Environmental and reproductive cues*

Animals use environmental stimuli or cues in order to make behaviourally relevant decisions such as locating food and mates, avoiding predators, and engaging in social interactions (Dusenbery, 1992). These cues can be visual, mechanical, or chemical and specialized sensory systems are required for detection of these cues. All animals detect and react to chemicals in the external environment and chemoreception plays an important role in guiding the behaviour of many animals (Hildebrand, 1995). Chemical cues are important in the aquatic environment as visual and auditory cues are often inefficient (Burks and Lodge, 2002). Furthermore, chemical cues in the aquatic environment are easily transmitted and have potential for high signal-to-noise ratios, increasing an animal’s ability to detect them (Wisenden, 2000). Chemical cues can be especially useful when the aquatic environment is turbid, for nocturnal animals, and for species with poorly developed visual systems (Wisenden, 2000; Breithaupt and Eger, 2002). Chemical cues can mediate behaviours like feeding, predator avoidance, swarming, migration, intraspecific communication and reproduction (Lonsdale et al., 1998). With respect to an animal’s fitness, reproductive cues may be the most critical use for chemical cues as they may be crucial for locating a receptive mate.

Reproductive cues are commonly used by aquatic animals for both mate localization and attraction, as well as for evaluating the reproductive state of the sender (Gleeson, 1991; Bouchard et al., 1996) and to synchronize reproductive behaviour (Haynes and Millar, 1998; Wyatt, 2003). Coordinating reproduction is very important, particularly for animals which must release gametes at the same time as their partner (Wyatt, 2003). The use of these cues for
reproduction involves individuals of one sex releasing odour(s), which subsequently attracts a sexually receptive conspecific. For aquatic animals, reproductive cues are generally comprised of mixtures of compounds and behavioural responsiveness to them is largely instinctual, sexually-dimorphic, and species specific (Sorensen, 1996).

The use of chemicals as reproductive cues may have evolved in animals as hormone(s) or other molecules being released into the environment and subsequently detected by chemoreceptors of a conspecific (Sorensen and Stacey, 1999). If detection of a particular chemical cue leads to greater reproductive success, there may be selection for receptors more sensitive to that cue or those receptors may be expressed in greater numbers (Wyatt, 2003). In some cases, animals may evolve a finely tuned system, including specialized sensory organs and brain circuits, to detect and respond to conspecific mating cues (Sorensen and Stacey, 1999). The importance of these reproductive cues for reproductive success is reflected in the chemosensory capabilities in many species of aquatic animals (Hildebrand, 1995).

Understanding of the neurobiology and organization of neural circuits of the olfactory system is crucial for relating properties of an odorant to an animal’s behavioural response to it (Hildebrand, 1995). Chemosensory information, including reproductive cues, is initially received at peripheral sensory receptors. Initially, odorants cross the lymph or boundary layer and bind to the extracellular surface of G-protein coupled receptors, located on the dendrites of bipolar neurons. Following this binding, a cascade of events is initiated that transforms the chemical energy of binding into a neural signal, thus changing the membrane potential (reviewed in Firestein, 2001; Rützler and Zwiebel, 2005). This sequence begins with the ligand-bound receptor activating the G-protein (Jones and Reed, 1989). The alpha subunit of the G-protein then detaches and, in turn, attaches to and activates adenylate cyclase (Pace and Lancet, 1986).
Once this occurs, abundant ATP is converted to cyclic AMP, a signaling molecule. Cyclic AMP then binds to the intracellular surface of an ion channel (a cyclic nucleotide-gated channel) and the channel opens, allowing an influx of Ca$^{2+}$ and Na$^+$ (Firestein et al., 1991). Inactive sensory neurons normally maintain a resting voltage of about -65 mV, but when the cyclic nucleotide-gated channels open, the influx of and Na$^+$ and Ca$^{2+}$ ions causes the inside of the cell to become less negative. If the channels are open for long enough, this causes the membrane potential to become approximately 20 mV less negative and the cell reaches threshold and generates an action potential. The action potential is then propagated along the axons to the higher order brain centers, including the olfactory lobe or bulb (reviewed in Firestein, 2001; Rützler and Zwiebel, 2005).

Olfaction and primary olfactory pathways are conserved across both aquatic and terrestrial, invertebrates and vertebrates (reviewed in Hildebrand, 1995; Firestein, 2001). Invertebrates and vertebrates display a roughly laminar olfactory bulb (vertebrates) or lobe (invertebrates) where olfactory receptor neurons converge into small regions of dense neuropil called glomeruli. From this, receptor neurons synapse with olfactory second-order projection neurons (Wilson, 2004). In crustaceans, olfactory receptor neurons of the antennules innervate only one glomerulus of the olfactory lobe (Schmidt and Ache, 1992; Mellon and Alones, 1993). The olfactory lobe is also closely associated with the accessory lobe (Sandeman and Scholtz, 1995; Sullivan and Beltz, 2004). Secondary projections from the olfactory lobe or accessory lobe may then synapse on higher order sensory-motor centers which may control motor behaviour (reviewed in Schmidt, 2007).
Crustacean reproductive cues

Crustaceans provide an ideal experimental model for studying chemoreception because they respond to many types of chemical cues in their environment and perform stereotypical behaviours in response to these odours. A growing body of research demonstrates that many types of decapod crustaceans use chemical cues for reproduction and mating (reviewed in Dunham, 1978, 1988), such as crabs (Ryan, 1966; Gleeson, 1980; Asai et al., 2000; Hardege et al., 2002), lobsters (Atema and Engstrom, 1971; Atema et al., 1979; Bushmann and Atema, 1997, 2000), and crayfish (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Stebbing et al., 2003a,b). Typically, female crustaceans secrete cues in their urine and this may attract mating partners (Bushman and Atema, 1996). With this, females may also increase urine output and current generation during reproductive pairings (Simon and Moore, 2007). Despite the fact that researchers have shown that mating cues, released in female urine, elicit precopulatory behaviours in conspecific males, very little is known about the structure or chemical composition of these cues used by crustaceans (Dunham, 1978, 1988; Bechler, 1995). However, the use of chemical cues to attract a mate is thought to be employed by crustacean species in which individuals experience difficulty finding a mate, such as those with a restricted breeding period and/or those found in areas of the aquatic environment where visual cues are limited (Salmon, 1983; Thorp, 1984).

Crayfish may rely on chemical cues from their environment for mating, as they are nocturnally active and may be found in heterogeneous/turbid habitats (Ackefors, 1996; Barbaresi and Gherardi, 2001). Specifically, crayfish may use chemical cues in order to locate sexually receptive conspecifics and synchronize mating (Thorp, 1984). These cues may be beneficial as
both male and female crayfish are found in reproductive and non-reproductive forms and these forms may temporarily overlap (Crocker and Bar, 1968; Berrill and Arsenault, 1984).

Both male and female crayfish are sexually dimorphic and moult into reproductive and non-reproductive forms depending on the time of year. These forms may be identified by examining their external reproductive morphology. Reproductive female crayfish contain glair glands which are located on the ventral side of the uropods. Glair is a mucus-like substance secreted during egg fertilization and may enable adherence of the eggs to the female during excretion (Holdich, 2002). Reproductive (form I) males can be differentiated from non-reproductive (form II) males by examining their copulatory stylets (Crocker and Barr, 1968). Form I males have relatively long, white copulatory stylets that extend to the base of the second pereiopods when the abdomen is flexed. The stylets of form II males differ from form I males in that they are shorter, yellow coloured, and are less structurally defined. Also, several species of crayfish exhibit morphological differences in their major chelae between reproductive forms. The major chelae of form I male crayfish are typically larger and more robust in relation to their body size when compared to form II males (Crocker and Barr, 1968; Stein, 1976).

The major chelae are important mechanical structures used for reproduction (Stein, 1976; Stein et al., 1977; Sneddon, 1990; Keller and Hazlett, 1996). Male crayfish use their major chelae for physical manipulation of females (mating stages 1-5, Stebbing et al., 2003a, 2004). Researchers have shown that male reproductive success is positively correlated with chelae length in crayfish (Stein, 1976) and that chelae loss negatively affects mating success (Sekkelton, 1988; Abello et al., 1994). For these reasons, it is thought that the most important purpose of the major chelae is their use in reproductive activities (Stein, 1976). Moreover, physiological studies have demonstrated that the major chelae of crayfish contain both mechano- and chemosensory
neurons (Bauer and Hatt, 1980; Hatt and Bauer, 1980; Altner et al., 1983). When male crayfish moults from form II to form I, the size of the major chelae increases. This increase may also lead to an increase in the chemosensory abilities of the major chelae (Stein, 1976; Snedden, 1990); however, there is very little information about the role that the major chelae play in chemoreception of mate cues.

*Crayfish mating behaviour*

Several studies have investigated the use of mating cues by female crayfish and their effects on conspecific male behaviour (see Table 1). These include attraction (Ameyaw-Akumfi and Hazlett, 1975; Gaudioso Lacasa and Cabello, 1979; Tierney and Dunham, 1982, 1984; Bechler et al., 1988; Stebbing et al., 2003a,b), attempted copulation (Gaudioso Lacasa and Cabello, 1979; Villanelli and Gherardi, 1998), decreased aggression (Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985; Dunham and Oh, 1992), increased motility (Acquistapace et al., 2002; Stebbing et al., 2003a), and increased time spent with a conspecific female conditioned water source (Stebbing et al., 2003a). Gaudioso Lacasa and Cabello (1979) demonstrated that male *Austropotamobius pallipes* went through five stages of attraction to female conditioned water, with the peak being when females were most receptive to copulation. Courtship and mating typically progress from an initial aggressive interaction between a pair to a ritualized submissive behaviour by the female, leading to copulation. Stebbing et al. (2003a) described seven distinct stages of mating behaviour in the crayfish (*Pacifastacus leniusculus*), including orientation, contact, seizure, turning, mounting, spermatophore deposition, and dismounting. Although these mating stages are likely similar among crayfish species, the duration of each process differs between individuals and species and may be regulated using reproductive cues.
Generally, male crayfish are attracted to female odours [e.g. *A. pallipes* (Gaudioso Lacasa and Cabello, 1979); *Orconectes propinquus* (Tierney and Dunham, 1982, 1984); *P. leniusculus* (Stebbing et al., 2003a,b); *P. clarkii* and *P. zonangularis* (Bechler et al., 1988)]. Female conditioned water has been shown to induce increased activity levels in reproductive male crayfish (Stebbing et al., 2003a). Male crayfish handle a female conditioned water odour source more when compared to a control (water) or conspecific male odours (Stebbing et al., 2003a). Also, male crayfish may become submissive, with their chelae down, in the presence of conspecific female odours (Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985; Dunham and Oh, 1992) and may attempt copulation with a receptive female or female odour source (Gaudioso Lacasa and Cabello, 1979; Villanelli and Gherardi, 1998; Stebbing et al., 2003a).

Aggressive same-sex behavioural displays (e.g., meral spread) are frequently observed in males and females (Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985; Dunham and Oh, 1992); however, these aggressive behaviours change when conspecific odours are released from individuals of the opposite sex. Both male and female *P. clarkii* showed submissive behaviours (major chelae down) when presented with odours of conspecifics of the opposite sex (Ameyaw-Akumfi and Hazlett, 1975; Dunham and Oh, 1992). However, odours from same sex conspecifics resulted in a continuation of aggressive posturing. Given the differential aggressive behaviours displayed by male and female conspecifics to conditioned water or individuals located within perforated test containers, chemical mating cues may function to alter aggressive behaviours and facilitate mating.

Reception of chemical cues, including mating cues, occurs via peripheral chemoreceptors located within sensory hairs (or setae). Chemical stimuli enter into the setae and reach the dendritic processes of sensory neurons by movement through pores in the cuticle covering the
setae (Laverick and Ardill, 1965). Sensory setae, both mechano- and chemosensory setae (see Figure 1), are typically located on the cuticle of chephalothoracic appendages, including the antennules and major chelae (Derby, 1982). Several studies have shown that the antennular aesthetasc hairs of crayfish are important for peripheral perception of mate odours (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992). When the lateral antennules were removed from crayfish (O. propinquus and P. clarkii), response to a female odour source decreased significantly (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992). More recently, a study by Corotto et al. (1999) in crayfish (P. clarkii) found that the antennules were not necessary for localization of females for the purpose of mating. When the crayfish had their antennules ablated, successful mating occurred in the same amount of time. This suggests that another sensory appendage, perhaps the major chelae, may also be important for the perception of female odours. Currently, there is very little information about the role that the major chelae play in chemoreception and their use in discrimination and localization of conspecific mating odours is currently unknown.

Dissertation overview and objectives

Currently the major chelae of form I male crayfish are thought to be used for the physical manipulation of mates during reproductive activities (Stein, 1976; Stein et al., 1977; Sneddon, 1990; Keller and Hazlett, 1996). However, evidence suggests that the major chelae may also be important chemosensory appendages, used for the perception of mate odours. The major chelae of crayfish have been shown to contain both mechano- and chemosensory neurons (Bauer and Hatt, 1980; Hatt and Bauer, 1980; Altner et al., 1983). Also, the localization of females by male crayfish and timing of reproductive activities are not impacted by the removal of the lateral antennules (Corotto et al., 1999), while the loss of the major chelae negatively affects mating
success (Sekkelton, 1988; Abello et al., 1994). Lastly, male crayfish handle a female conditioned odour source significantly more with their major chelae when compared to water control (Stebbing et al., 2003a). Taken together, these studies suggest that the major chelae of male crayfish may be used to discriminate female odours. It was thus hypothesized that male crayfish will use their major chelae for discrimination and localization of a conspecific female odour source. The goals of this dissertation were to: 1) investigate whether the major chelae of male crayfish (*O. rusticus*) are important chemosensory appendages necessary for the detection of female odours and if so, whether there are differences between reproductive forms, 2) determine if there are differences in chelae morphology between reproductive forms, and 3) elucidate if male crayfish perform sampling behaviours with their major chelae when presented with female odour. Overall, this research will determine if the major chelae are important for the localization and discrimination of female odours and will determine which sensory structures are responsible for this discrimination. This research will also provide insight on the importance of the major chelae for reproductive behaviours such as mate recognition and will lead to new insights on peripheral processing of mate odours.

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1Portions published as: Corkum, L.D. and Belanger, R.M. 2007. Use of chemical communication in the management of freshwater aquatic species that are vectors of human diseases or are invasive. *General and Comparative Endocrinology, in press.*
CHAPTER II.
THE USE OF THE MAJOR CHELAE BY REPRODUCTIVE MALE CRAYFISH
(ORCONECTES RUSTICUS) FOR DISCRIMINATION OF FEMALE ODOURS

Introduction

Crustaceans are known to utilize chemical communication for many aspects of their lives. Many behaviours of crustaceans have been shown to be mediated through chemical senses; these include: food acquisition and orientation (Moore et al., 1991; Kraus-Epley and Moore, 2002), conspecific recognition (Copp, 1986; Schneider and Moore, 2000), identification of conspecifics and/or social status (Schneider et al., 2001; Bergman et al., 2003), identification, localization, and sex recognition for mating purposes (Dunham and Oh, 1992; Stebbing et al., 2003a), and detection of alarm cues (Hazlett, 1990, 1994; Schneider and Moore, 2000) and predators (Willman et al., 1994; Keller and Moore, 1999). Because crustaceans and other aquatic animals are considered ‘leaky bags’ (Atema, 1986), the chemicals they release into the environment may contain information about their internal states. This creates the potential for either active chemical communication with the sender actively releasing chemical information to convey information to the receiver or passive information released via normal metabolic processes. In either case, information about an animal’s internal physiological state, including reproductive cues, could be transmitted to the receiving animal.

In crustaceans, reception of chemical cues occurs via peripheral chemoreceptors located within sensory hairs (or setae). Chemical stimuli reach the dendritic processes of sensory neurons by movement through pores in the cuticle covering the setae (Laverick and Ardill, 1965). Sensory setae are typically located on the cuticle of chephalothoracic appendages (Derby, 1982). These appendages, containing both mechano- and chemosenory setae, include antennae, antennules, maxillipeds (mouthparts), and periopods (major chelae and walking legs). The most studied appendage, the antennules, are thought to be used for long distance orientation to prey items (Derby et al., 2001) and mate odours (Dunham, 1978; Gleeson, 1980; Kamio et al., 2005). In contrast, maxillipeds and walking legs have been found to respond to food odours (Derby and Atema, 1982; Corotto and O’Brien, 2002; Garm et al., 2005). Sensory receptors found on the walking legs can also function in detecting nearby food (Derby and Atema, 1982; Corotto and O’Brien, 2002), but have also been implicated as potential sources of information for distance orientation (Moore et al., 1991; Keller et al., 2003). Major chelae of crayfish contain both simple and plumose setae. Simple setae have been demonstrated in crayfish and other crustaceans as having a bimodal chemo- and mechanosensory function (Weisbaum and Lavalli, 2004; Obermeier and Schmitz, 2004; Belanger et al., 2008), while plumose setae are thought to be mechanoreceptors (Tautz et al., 1981). Currently, there is very little information about the role the major chelae play in chemoreception, although observations from our laboratory suggest that the chelae may be involved in the detection of female odours.

Previous studies have attempted to isolate the sensory appendage responsible for peripheral perception of mate odours in crustaceans (Ameyaw-Akumfi and Hazlett, 1975; Dunham, 1978; Gleeson, 1980; Tierney et al., 1984; Kamio et al., 2005). In the crab, it is believed that the lateral antennule is responsible for pheromone detection (Dunham, 1978;
Gleeson, 1980; Kamio et al., 2005). When this appendage was removed, response to a female odour source decreased significantly (Kamio et al., 2005). Similar results were demonstrated in two crayfish species, *Orconectes propinquus* and *Procambarus clarkii* (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984). However, a study by Corotto et al. (1999) in crayfish (*P. clarkii*) did not find that the antennules are necessary for localization of females for the purpose of mating. When the crayfish had their antennules ablated, successful mating occurred in the same amount of time. These studies have ignored the potential role of the major chelae as chemoreceptive organs used for detection of conspecific odours.

Chelae are known to be important mechanical structures used for reproduction in crustaceans. In the freshwater prawn (*Macrobrachium rosenbergii*), males with larger chelae are more successful at courting and mating females (Ra’anan and Cohen, 1985; Ra’anan and Sagi, 1985). In crayfish, the major chelae are needed for the physical manipulation of females (stages 1-5, Stebbing et al., 2003a). Because male reproductive success has been positively correlated with chelae length in crayfish, researchers have concluded that the most important purpose of the major chelae is their use in reproductive activities (Stein, 1976). Physiological studies on the major chelae of crayfish have indicated that the chelae have both mechano- and chemosensory neurons (Bauer and Hatt, 1980; Hatt and Bauer, 1980; Altner et al., 1983). Some species of crayfish moult from reproductive to non-reproductive forms and this may alter both the mechanical and sensory capabilities of the major chelae (Stein, 1976; Snedden, 1990).

Male *Orconectes rusticus* are dimorphic in that they moult continuously from season to season, displaying two different morphotypes (Crocker and Barr, 1968). The reproductive form differs from the non-reproductive form in that it has long white reproductive stylets and more robust major chelae in proportion to body size (Crocker and Barr, 1968; Stein, 1976). Belanger et
al. (2008) have also shown that form I crayfish have a larger proportion of sensory hairs (plumose and simple setae) lining the dorsal surface of the major chelae (Figure 2). This sensory hair difference coincides with seasonal differences as sensory hairs are more abundant during the reproductive season. These findings create the possibility that male crayfish may use one or both of these sensory hairs types on the major chelae for mate localization, however this has not been tested behaviourally.

The goal of our study was to determine whether reproductive male crayfish, *O. rusticus* (Girard, 1852), use chemo- or mechanosensory structures, located on their major chelae, to detect and discriminate conspecific female cues. Stebbing et al. (2003a) showed that male crayfish handled (made contact with, seized, or mounted) a female odour source more than the water; however their study did not investigate the sensory appendage responsible for the perception of female odours. To elucidate what role the major chelae have in the perception of female odours, we used a behavioural bioassay to test this. Because it is known that the crustacean chemoreceptive system has the ability to discriminate many chemicals and chemical mixtures (Ache et al., 1976; Carr and Derby, 1986), it is important to decipher which peripheral appendage is important for distinguishing conspecific odours. Eventually, this will lead to an understanding of the peripheral processing of mate odours, as well as higher-order brain processing centres that are important for mate recognition.
Materials and Methods

*Animals*

*Orconectes rusticus* (females, form I, and form II males) were collected by seining in the Portage River near Bowling Green State University in Bowling Green, Ohio, USA. Intermoult male crayfish used in experiments were visually and mechanically isolated in plastic flower pots that contained lids with holes (17.8 cm diameter and 9 cm depth) that were stored in a flow-through holding tank (48 × 154 × 31 cm) where water within the pots was continuously exchanged with water in the tank. The holding tank was in an environmental chamber (23°C, 14:10 hour light dark cycle). Crayfish males were held in population tanks with other males for 1 week to 5 months prior to being isolated for at least 48 h before behavioural experiments. Crayfish mass, carapace, and chelae lengths (±SE) were measured for form I and form II males used in this experiment (form I – 18.73 ± 0.63 g; 3.63 ± 0.04 cm carapace length; 3.70 ± 0.06 cm chelae length, form II – 21.43 ± 0.84 g; 3.87 ± 0.05 cm carapace length; 3.83 ± 0.08 cm chelae length) and only crayfish with intact appendages were used (i.e., antennae, lateral and medial antennule filaments, chelae, maxillipeds, and walking legs). As in Stein (1976), form I males had significantly larger major chelae in proportion to carapace length (p = 0.006, t = 2.781, df = 318, N = 320; *t*-test). Crayfish were fed a diet of rabbit pellets three times per week. Experiments using form I males were completed between August and October 2003-2005 and experiments using form II males were completed between February and July 2004. Form I and form II individuals were stored separately, as their availability was seasonal. All treatments were performed between 9.00 and 18.00 h.

Form I and form II males were identified by examining their reproductive stylets (Crocker and Barr, 1968). *O. rusticus* form I males have relatively long reproductive stylets that
extend to the base of the second pereiopods when the abdomen is flexed. The stylets of form II males differ from form I males in that they are shorter, yellow coloured, and are less structurally defined.

Conditioned water stimulus and setup

To examine male attraction to a female odour source and consequently chelae odour sampling ability, fresh female-conditioned water was obtained using a procedure similar to Stebbing et al. (2003a). Conditioned water was obtained from six reproductive female *O. rusticus* (9.79 ± 0.61 g and 3.22 ± 0.10 cm carapace length) by holding them individually in clear plastic pots (40 × 20 × 25 cm) containing 500 ml of aerated dechlorinated water for a 24-hour period. Water from each pot was then collected, combined, and filtered (Whatman® 185 mm #1004185) to remove debris and stored at −20°C until used. Conditioned water from 6 male (form I and form II) *O. rusticus* (11.07 ± 1.08 g and 3.32 ± 0.11 cm carapace length) was also obtained, filtered, and stored using the same method. Different plastic pots were used for the collection of each odour type so that there was no cross contamination of the odours collected. There was no difference in mass or carapace length between the female and male crayfish odour donors used in this experiment (p = 0.322, t = 1.042, df = 10, N = 12; t-test). The negative control for this experiment was fresh dechlorinated water that had been aerated over a 24-hour period with no crayfish present. The water was then subsequently filtered in a similar manner to the previous conditioned water treatments. A positive control of filtered fish homogenate (food odour) was prepared fresh daily in a similar manner to Kraus-Epley and Moore (2002) by weighing 50 g of Pollock fish fillets pureed in a blender with 500 ml of dechlorinated water. This homogenate was then filtered as with all the other odours tested in this experiment.
**Blocking protocol**

The treatment groups consisted of the following: (i) Form I males with intact chelae; (ii) Form I males with chelae blocked; (iii) Form II males with intact chelae; (iv) Form II males with chelae blocked. Twenty individuals were used in each of the four treatment groups and were exposed to one of the four randomized odours: female-conditioned water, male-conditioned water, dechlorinated water (negative control) and filtered fish homogenate (positive control). Each animal was used only once and a total of 320 crayfish (4 treatments × 4 odours × 20 crayfish) were used in this study. The blocking procedure, similar to Kraus-Epley and Moore (2002), was performed by restraining crayfish and covering the sensory hairs on their major chelae with superglue (Duro Quick Gel®). Using the applicator tip, glue was applied to the dorsal surface of chelae of experimental animals beginning at the base and extending to the tip, covering the sensory hairs (see Figure 2). Afterwards, a drying accelerator (Zip Kicker™) was applied to the superglue with a cotton swab in order to speed drying time. The dorsal surfaces of the major chelae were blocked because Solon and Cobb (1980) showed that lobsters (*Homarus americanus*) tend to position themselves so that the dorsal surfaces of the chelae would be more likely to receive stimulus events. Blocking animals by covering receptors with super glue inhibits both chemo and mechanoreceptors (Kraus-Epley and Moore, 2002; Bergman et al., 2003). Intact animals had a similar amount of glue placed at the base of the carapace (on their back) to ensure that changes in behaviour were not due to the presence of superglue. Chelae of intact animals were washed with tank water using a syringe to simulate the physical stimulation of the chelae of experimental individuals during gluing. When the glue was completely dry (~2 min), each individual crayfish was placed in the test chamber for the acclimation period.
**Experimental trials**

Blocked and intact \((N = 20\) trials per treatment) form I and form II male *O. rusticus* were placed in opaque, visually isolated tanks \((25 \times 14 \times 14.5\) cm). Each tank contained 2 litres of fresh dechlorinated water that was not aerated during recording periods. Males were allowed to acclimate for 1-hour to the test tank before the start of the experiment. As in Stebbing et al. (2003a), the test tank contained a blue cylindrical aquarium air stone 2 cm in length attached to 30 cm of 5 mm-diameter silicone tubing. A syringe containing 20 ml of the test odour was attached to the air stone via 15 cm of 5 mm-diameter silicone tubing. Between trials, each of the test tanks and air stone odour sources were rinsed vigorously with distilled water. Different air stones and syringes were used for each odour type in order to prevent cross contamination. The male’s air stone handling time (odour source handling) was recorded for 15 minutes before the introduction of the treatment odour (pre-test period), and for another 15 minutes afterwards (test period) using a Canon XL-1 (digital video camera) mounted above the test tank. All odours were introduced by hand to the test arena at a rate of approximately 5 ml/s after removing the air from the tubing using negative pressure on the syringe. Preliminary dye trials demonstrated that the stimulus delivery procedure did not allow the odour to rapidly mix. Instead, a gradient was created from the diffusion of the odour away from the odour source. Experimental setup and recording periods for these bioassays were similar to those of Stebbing et al. (2003a) and Belanger et al. (2006, 2007).

**Data collection and analysis**

‘Handling’ of the odour source was defined as the animal making contact with, seizing, or mounting the air stone (Stebbing et al., 2003a). As in Schmidt and Derby (2005), odour source handling time before the addition of odours in the 15-minute pre-stimulation period was
subtracted from post-stimulation period in order to subtract the baseline activity from activity induced by stimulation. This data was analysed for normality and was assessed using a 3-way ANOVA with a Fisher LSD post-hoc test to look for differences across treatments. With this, overall handling responses comparing blocking status, odour type presented, and the reproductive form of the crayfish were compared. For those treatments that showed significant responses to the odour, appendage handling of the odour source was further analysed to differentiate these responses. For intact form I treatments with female-conditioned water stimulus, intact and blocked form I and form II’s treatments with filtered fish homogenate stimulus, handling of the odour source with chelae/first walking legs and maxillipeds/first walking legs was recorded. We used data which examined chelae/first walking legs and maxillipeds/first walking legs because as soon as the major chelae contacted the odour source, walking legs were engaged within 3 seconds; this was the same for maxillipeds. For this reason, analysing individual chelae and maxillipeds handling was not a sufficient indicator of sensory appendage use. Being that we include walking leg data for examining both, the major chelae and maxillipeds, the use of walking legs for chemoreception of food and mate odours can be excluded. Appendage handling times were analysed separately using a 1-way ANOVA with a Fisher LSD post-hoc test to look for differences across treatments.
Results

Odour source handling

(1) Female-conditioned water responses
Intact reproductive (form I, FI) male crayfish responded to reproductive female-conditioned water with an odour source handling time of 46.35 ± 17.00 seconds (sec) [mean ± standard error (SE)]. Blocked form I males spent significantly (p = 0.018) less time (8.00 ± 3.80 sec) handling the female-conditioned water odour source when compared to intact form I males (Figure 3A). Intact and blocked non-reproductive (form II, FII) male crayfish did not respond to female-conditioned water (−0.75 ± 2.91 sec and 1.50 ± 3.25 s, respectively) and handled the female odour source significantly less (p = 0.004 and p = 0.006, respectively) than intact form I males (Figure 3A). Female odour source handling times were similar when form I sensory-blocked individuals were compared to both form II treatments, intact, and blocked (p = 0.587 and p = 0.687, respectively). There was also no difference (p = 0.703) when blocked and intact form II treatments were compared. Therefore, the manipulation of the odour source by form I crayfish was eliminated when males had had their major chelae sensory-blocked, making blocked form I male responses comparable to those of form II males.

(2) Male-conditioned water responses
When both intact form I and form II males were exposed to conspecific male-conditioned water, there were no significant differences observed when compared to blocked individuals of the same form. Intact form I males spent 9.95 ± 12.31 sec handling the male-conditioned water odour source while blocked individuals spent 6.45 ± 5.05 sec (p = 0.828; Figure 3B). The data for form II males was also not significantly different (p = 0.850) where intact males spent 0.95 ±
5.81 sec handling the male-conditioned water odour source and blocked males handled the source for 4.00 ± 3.38 sec (Figure 3B).

(3) Food odour responses
When filtered fish homogenate, food odours (positive control), was tested, both intact and blocked form I male crayfish responded positively to food odours with increased handling time of the odour source. Intact form I males handled the odour source for 32.95 ± 14.71 s, while blocked individuals handled the odour source for 49.00 ± 22.39 sec (Figure 3C). These did not differ significantly from one another (p = 0.760). Form II males handled the filtered fish homogenate similarly (p = 0.175) with intact males spending 68.60 ± 15.96 sec handling the odour source and those males with their major chelae blocked handling the odour source for 46.70 ± 19.45 sec (Figure 3C). There was no difference across these treatments demonstrating that the chelae blocking did not affect responses to food odours.

(4) Water responses
When exposed to water (negative control) intact form I male crayfish spent −6.90 ± 6.43 sec handling the odour source while blocked crayfish handled the water source for 4.30 ± 3.15 sec (Figure 3D). Odour source handling times were not significantly different (p = 0.821). For form II crayfish, there was no difference (p = 0.487) between the time intact males spent handling the water source (8.35 ± 10.94 s) and blocked males (4.70 ± 3.64 s; Figure 3D). Overall, there was no significant difference between all of these treatments.

Comparison across treatments
Blocking of the major chelae significantly altered the response of form I males to female-conditioned water, but did not alter the response to fish homogenate (F_{(3,298)} = 15.54, p < 0.0005; 3-way ANOVA). Intact form I males responded significantly to both female-conditioned water
and to fish homogenate (odour source handling time >25 s), while blocked form I males only responded to fish homogenate and not female odours. Blocking the major chelae of form I males thus had an impact on the response by form I male crayfish to female odours however; this sensory-blocking technique did not affect their response to food odours. When comparing all treatments, odour source handling times were similar for form I blocked male crayfish to female odours, both intact and blocked form II males to female-conditioned water, all crayfish exposed to conspecific male odour, as well as all test animals exposed to water.

**Odour source handling by different appendages**

For those five treatments that showed significant increases in handling time (>25 s) to the odour source [intact form I males to food odour (IFIF), blocked form I males to food odour (BFIF), intact form II males to food odour (IFIIF), blocked form II males to food odour (BFIIF), and intact form I males to female odour (IFIFe)], video footage was then further analysed to determine if there was a difference in the usage of sensory appendage for manipulation of the source. For those treatments, handling of the odour source with the chelae/first walking legs and maxillipeds/first walking legs was compared. This was done in order to determine if different appendages engaged the odour source when female and food odours were presented.

When presented with female-conditioned water, intact form I males used their chelae/first walking legs significantly more ($F_{(4,94)} = 3.671; p = 0.008$) to handle the odour source (Figure 4A). Overall, intact form I male crayfish handled the female odour source with their chelae/first walking legs for $20.30 \pm 10.32$ sec (43.8% of total handling time). The response to female odour from intact form I males was statistically different ($p = 0.002$) from form I intact males response to food odours. Those individuals did not respond to food odours with their chelae/first walking legs ($-1.15 \pm 1.44$ s, -3.5%). Blocked form I males handled the food odour source for $0.25 \pm$
0.86 sec (0.5%) with their chelae/first walking legs. This differed from the time intact form I individuals spent handling the female odour source (p = 0.003). Form II (intact and blocked) male crayfish also spent very little time handling the food odour source with their chelae/first walking legs also. Intact form II males handled the odour source for 1.25 ± 0.73 sec (1.8% of total time) while blocked form II males spent 0.80 ± 0.56 sec (1.7% of total time) handling the food odour. This differed significantly from the time spent by intact form I males responding to female odour (p = 0.005 and p = 0.004, respectively).

Data from maxillipeds/first walking legs handling of the odour source was also examined (F(4, 95) = 1.785; p = 0.138) (Figure 4B). These data demonstrate that there is an increase handling of food odour sources with maxillipeds/first walking legs. Intact form I males handled a food odour source for 20.37 ± 10.29 sec (61.8% of total time) while blocked form I individuals spent 42.20 ± 20.34 sec (86.1%) handling the food odour source. Intact and blocked form II males handled the fish homogenate odour source for 40.00 ± 11.54 sec (58.3%) and 24.85 ± 12.33 sec (53.2%), respectively. When female odour was presented to intact form I males, they handled this odour source for −0.40 ± 5.21 sec with the maxillipeds/first walking legs. Overall, there was an increase handling time of female odours with chelae/first walking legs (Figure 4A) while food odours were handled mainly with maxillipeds/first walking legs (Figure 4B).
Discussion

The importance of the major chelae for the detection of female odours has been clearly demonstrated by the decrease in odour source handling by reproductive male *O. rusticus* following sensory-blocking of the setae on the major chelae. These results show that peripheral chemosensory input from the major chelae is required for the odour source grasping behaviour, rather than from receptors located on the lateral antennules (Tierney et al., 1984; Kamio et al., 2005). The deleterious effect of the blocking technique on the odour source handling behaviour is supported by previous observations on the loss of chemosensory mediated behaviour following this type of blocking in *O. rusticus* (Kraus-Epley and Moore, 2002; Bergman et al., 2003) and subsequent blockage of sensory receptors. Using the blocking technique allowed us to clearly demonstrate that only reproductive male crayfish use their major chelae for detection of female odours. Also, we have found that crayfish can differentiate female odours from others, including food odours, utilizing different sensory appendages for handling of food odours. Differences in odour source handling, along with the fact that reproductive male crayfish have proportionally more sensory hairs on their major chelae (Belanger et al., 2008), strongly suggests that the major chelae are chemosensory appendages that play a role in courtship and mating.

Both mechanical and chemical information presented to the major chelae may be important for mating and courtship activities. Courtship behaviour in crayfish, as described by Stebbing et al. (2003a), includes orientation, contact, seizure, turning, mounting, spermatophore deposition, and dismounting. During this orientation phase, male crayfish have been observed to wave their chelae horizontally through the water column in an odour-mediated sampling behaviour (T. Keller, pers. comm.). Chela waving behaviour has also been reported in *P. clarkii* by Itagaki and Thorp (1981) and it has been suggested by Dunham and Oh (1992) that chelae
waves represent potential sex discrimination with this behaviour. Chela waving behaviour is believed to be a chemosensory event as chelae have been shown to have chemoreceptive properties (Bauer and Hatt, 1980; Hatt and Bauer, 1980; Altner et al., 1983) and crayfish exhibit movements of their chelae similar to other appendages (e.g., antennule flicking) that have been shown to be associated with chemosensory sampling (Kraus-Epley and Moore, 2002). Finally, in our study, only reproductive forms of male crayfish exhibit this waving behaviour reactivity to female odours (Figure 3A). Taken together, these results indicate that the major chelae of reproductive male crayfish are chemosensory appendages used for the discrimination of female odours.

As well as being chemosensory appendages, the major chelae are also important physical structures that aid in reproduction in crustaceans (reviewed in Mariappan et al., 2000). It has been shown that form I male crayfish (O. propinquus) with large chelae are more successful in copulating with females than those with small chelae (Stein, 1976). Also, Snedden (1990) showed that male crayfish (O. rusticus) with larger chelae are better able to secure and orient females for copulations (stages 1-5, Stebbing et al., 2003a). It has been found that mating is unsuccessful in shore crabs (Carcinus maenas) if one or both of the major chelae are not functional (Sekkelton, 1988). In the freshwater prawn (M. rosenbergii), males with different chelae morphotypes have varying access to mates with individuals displaying larger chelae effectively courting mates (Ra’anana and Cohen, 1985; Ra’anana and Sagi, 1985). Our data suggest that chemosensory function of the major chelae in mating may also be important for explaining some of the previous findings.

Several species of crayfish have been reported to be able to discriminate between male and female odours (e.g., P. clarkii, Dunham and Oh (1992); Ameyaw-Akumfi and Hazlett
(1975); *O. virilis*, Hazlett (1985); *O. propinquus*, Tierney et al. (1984)) presumably through the use of sex pheromones. In studies concerned with the detection of mate odours in crayfish, researchers have focused on the antennules, but have found conflicting results (e.g., Corotto et al., 1999; Tierney et al., 1984). Tierney et al. (1984) demonstrated that *O. propinquus* perceive conspecific odours with their antennules, whereas Corotto et al. (1999) found that antennules were not necessary for localization and mating in *P. clarkii*. The contradictory results in regards to antennule use in these two studies may be explained by our results showing the potential role of the major chelae in the detection of female odours. Stebbing et al. (2003a) showed that male crayfish (*Pacifastacus leniusculus*) are attracted to a conspecific female odour source and handle this source with their major chelae, however they did not investigate which sensory appendage was important for this response. Our data coincide with data presented by Stebbing et al. (2003a) and Corotto et al. (1999) by showing that the major chelae are necessary for the discrimination of female odours in reproductive male crayfish. This was not the case however for food odours (positive control) where both sensory-blocked and intact reproductive and non-reproductive males still responded to food odours presented to them (Figure 3C), suggesting alternate sensory appendage(s) may be used for perception of food odours.

Overall, crayfish use chemical cues for many types of behaviours that are important to survival and reproductive success (Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985; Tierney and Atema, 1988; Dunham and Oh, 1992, 1996; Corotto et al., 1999; Moore and Grills, 1999; Giri and Dunham, 2000; Stebbing et al., 2003a). Odours from female crayfish are perceived and processed peripherally by the major chelae of reproductive male crayfish. These odours may be recognized by non-reproductive males; however, they, like sensory blocked reproductive males, do not behaviourally respond to the odour source. Therefore the attraction to female odour by
reproductive males may be a conspecific mating cue or pheromone used for reproductive purposes. Male crayfish, regardless of reproductive state, do not respond to conspecific male odours (mixed form I and form II). This is not surprising as previous work on naïve male crayfish shows that these males do not respond to a naïve male odour source (Bergman and Moore, 2005). This indicates that the response by reproductive male crayfish to the female odour source is not a general response to conspecifics, rather it is sex-related however, pure form I and form II odours must be analysed separately to elucidate if male crayfish can use chelae chemoreceptors to discriminate male reproductive status. Our data suggest that there is peripheral processing of conspecific female odours by the major chelae and that they use these large sensory organs not only to aid mechanically in reproduction, but also for chemoreceptive purposes. Coupled with this research, future work on major chelae sensory responses to stimuli will lead to insight on how the brain processes and integrates chemosensory information from multiple sensory appendages. This will ultimately lead to a clearer understanding of how organisms make decisions about their environment.
CHAPTER III.

SENSORY SETAE ON THE MAJOR CHELAE OF MALE CRAYFISH, ORCONECTES RUSTICUS (DECAPODA, ASTACIDAE): IMPACT OF REPRODUCTIVE STATE ON FUNCTION AND DISTRIBUTION

Introduction

Crustaceans are covered with rigid exoskeletons and receive external sensory input through cuticular hair organs referred to as setae, located on sensory appendages (Laverack, 1988; Derby, 1989). Setae are found over the entire surface of crustaceans, including the major chelae, and serve a wide range of mechano- and chemosensory functions (Felgenhauer and Abele, 1983; Watling, 1989). In particular, the major chelae of crustaceans have been shown to contain both mechano- and chemosensitive setae and may contain cuspidate (also referred to as toothed, squat, or fringed setae), hemate, smooth and plumose setae (e.g. Shelton and Laverack, 1968, 1970; Thomas, 1970; Derby, 1982; Weissberg and Derby, 1995; Lavalli and Factor, 1995; Vogt and Tolley, 2004; Belanger and Moore, 2006). Physiological studies on the major chelae of crayfish have indicated that the chelae have both mechano- and chemosensory neurons (Bauer and Hatt, 1980; Hatt and Bauer, 1980; Altner et al., 1983).

Several species of crayfish exhibit morphological differences in their major chelae between reproductive forms. Chelae in reproductive male crayfish are more robust in

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relation to their body size (Crocker and Barr, 1968; Stein, 1976). Most studies on the major chelae of crayfish have focused on their mechanical function during reproduction (Stein, 1976; Stein et al., 1977; Sneddon, 1990; Keller and Hazlett, 1996), although a recent study has shown that the major chelae are used to detect and discriminate female odours in reproductive (form I) male crayfish. Non-reproductive (form II) males do not show this ability (Belanger and Moore, 2006). All of these findings suggest that the major chelae are important structures for mating (Stein, 1976; Keller and Hazlett, 1996; Belanger and Moore, 2006). The goal of the present study is to complete a morphological and comparative analysis of the sensory setae found on the major chelae of form I and form II male crayfish, *Orconectes rusticus* (Girard: 1852). A comparative analysis of the chelae of both reproductive forms will allow us to determine if there is a quantitative difference in the sensory setae between the two reproductive forms, ultimately providing a link between the morphology and reproductive behaviour.

Numerous types of chemo- and mechanosensory setae have been morphologically described in decapod crustaceans (Figure 1). Chemosensory setae contain a terminal pore (Schmidt and Gnatzy, 1984) or thin cuticle at the distal tip when no pore is present (Tierney et al., 1986) and are permeable to water-soluble dyes (Slifer, 1960; Gleeson, 1982; Altner et al., 1983; Tierney et al., 1986; Cate and Derby, 2002a). These sensory setae contain chemosensory dendrites found within the setae that extend along the length of the setae, reaching the pore or distal tip (McIver, 1975; Altner et al. 1983) and typically lack setules (Ball and Cowan, 1977). Mechanosensory setae are mounted on a flexible membrane and have an abundant amount of setules, which increases their surface area and makes them sensitive to vibrations (Ball and Cowan, 1977; Felgenhauer and Abele, 1983; Crouau, 1981; Jacques, 1989; Watling, 1989). Dendrites do not extend into the setae, but are located at the base of these setae and are attached
to one side of the setae or the other (McIver, 1975; Ball and Cowan, 1977; Altner et al. 1983; Felgenhauer and Abele, 1983; Gill, 1986). Taken together, chemosensory setae should contain a terminal pore and be permeable to dyes. They will be innervated with dendrites that extend to the terminal pore, while mechanosensory setae will not have these features. However, many types of setae are bimodal chemo- and mechanoreceptors and will contain a combination of the features present in both chemosensory and mechanosensory setae (Jacques, 1989). Examining the internal and external morphology of the sensory setae of the major chelae allows us to determine putative sensory function.

The dorsal surface of the major chelae of male crayfish (*O. rusticus*) is lined with both smooth (or simple) and plumose setae arranged in discrete pockets (see Fig. 1, Belanger and Moore, 2006). In the crayfish *Austropotamobius pallipes*, smooth setae are described as having a smooth outline with pointed tips and an apical terminal pore (Thomas, 1970). Smooth setae have been previously demonstrated as having a bimodal chemo- and mechanosensory function in crayfish and other crustaceans (Weisbaum and Lavalli, 2004; Obermeier and Schmitz, 2004). Plumose setae are feather-like, with long setules coming off the shaft in two opposite rows, and contain a smooth pointed tip (Thomas, 1970). It has been shown that plumose setae of the antennules are non-innervated mechanoreceptors (Bender et al., 1984). Previous behavioural studies using sensory blocking technique indicated that only the smooth and plumose setae were necessary for female odour recognition by male *O. rusticus* (Belanger and Moore, 2006).

Several studies have shown that the antennular aesthetasc hairs of crustaceans are also important for peripheral perception of mate odours in crustaceans (Ameyaw-Akumfi and Hazlett, 1975; Gleeson, 1980; Tierney et al., 1984; Dunham and Oh, 1992; Kamio et al., 2005). When the lateral antennules were removed from helmet crabs (*Telmessus cheiragonus*), response to a
female odour source decreased significantly (Kamio et al., 2005). In crayfish (*O. propinquus* and *Procambarus clarkii*), similar results were demonstrated (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992). More recently, a study by Corotto et al. (1999) in crayfish (*P. clarkii*) did not find that the antennules are necessary for localization of females for the purpose of mating. When the crayfish had their antennules ablated, successful mating occurred in the same amount of time. Taken together, results of these studies and those obtained by Belanger and Moore (2006) suggest that chelae receptors may just provide additional chemical feedback necessary for discrimination and localization of a female odour source. Therefore understanding the morphology and distribution of sensory structures on the major chelae will be beneficial to the understanding of female odour source response and discrimination.

The aim of this study was three-fold. First, we quantified the number and spatial distribution of plumose and smooth setae on the major chelae of male crayfish, *O. rusticus*. Second, we performed a comparative analysis across the different reproductive forms (form I and II) to determine any changes in distribution with reproductive state. Finally, a series of morphological studies were performed to determine the putative sensory function of each seta type. Overall, this work will allow us to determine if there are any morphological differences in the major chelae of form I and II male *O. rusticus* that may account for the differences in chemosensory responses to female odours seen in previous behavioural studies (Belanger and Moore, 2006).
Materials and Methods

Crayfish Collection and Housing

Reproductive (form I) and non-reproductive (form II) male *Orconectes rusticus* were collected from the Portage River near Bowling Green State University in Bowling Green, Ohio, U.S.A. Intermoult male crayfish used in all imaging experiments were housed in population tanks in an environmental chamber (23°C, 14 light: 10 dark). Crayfish were fed a diet of rabbit pellets three times per week. Imaging experiments were completed between September 2004 and October 2005. Scanning electron microscopy was completed between January and April 2005. Crayfish mass, carapace length, and chelae length (mean ± S.E.) were measured for form I and form II males (form I – 16.76 ± 1.17 g; 3.50 ± 0.09 cm carapace length and 3.48 ± 0.09 cm chelae length; form II – 13.46 ± 2.01 g; 3.40 ± 0.19 cm carapace length and 3.28 ± 0.21 cm chelae length). For all imaging experiments, major chelae were dissected from the body of the crayfish after they were anesthetized in an ice bath for at least 30 minutes.

Form I and form II males were differentiated by examining their stylets (Crocker and Barr, 1968). Form I males were identified as having relatively long white stylets that extend to the base of the second pereiopods. The stylets of form II males differ from form I males in that they were shorter, yellow coloured, and less structurally defined.

Scanning Electron Microscopy

To examine the structure and sensory setae distribution on the dorsal surface of the major chelae, scanning electron microscopy was performed using a procedure modified from Cate and Derby (2002a,b). After dissection, major chelae of crayfish, form I and II (N = 5 of each form) were immediately placed in a sonicator containing 0.1 M phosphate buffered saline (PBS, pH 7.4) to remove debris from the chelae and sensory setae. After sonicating, chelae were fixed for
at least 48 hours in 2.5% gluteraldehyde/3% paraformaldehyde (PFA) in 0.1 M PBS. Following fixation, the chelae were rinsed for 10 minutes in 0.1 M PBS, post-fixed in 1% osmium tetroxide for 2 hours, and rinsed again for 10 minutes with 0.1 M PBS. Following rinsing, chelae were dehydrated in a graded ethanol series (40-60-80-95-100-100-100%; 15 minutes each step) and stored for final drying in 100% ethanol. Drying was accomplished by submerging the chelae in a graded hexamethyldisilazane (Electron Microscopy Sciences, Fort Washington, Pennsylvania) and ethanol series (Nation, 1983). After the last change of hexamethyldisilazane (100%) was complete, the tissue was allowed to air-dry overnight. The major chelae were then mounted onto stubs with double-sided tape and graphite paint, sputter-coated with gold/palladium, and examined using a scanning electron microscope (Hitachi S-2700 SEM). Sensory setae counts were performed and images were captured.

Sensory Setae Quantification and Data Analysis

While examining video images of the major chelae on the scanning electron microscope, the pockets of sensory setae present on the dorsal surface were quantified, as well as the number of individual sensory setae contained within each of these pockets. On each chela, the number of setae pockets and individual sensory setae within the pockets were quantified from the distal tip of the chelae to where the proximal part of the biting edge ended. Dividing the total number of pockets by the length of each particular chela normalized the number of setae pockets counted. This was done because there are size differences in the chelae between reproductive forms (Crocker and Barr, 1968) and number of pockets is correlated with length (Belanger, personal observation). A t-test was used to compare the overall number of setae pockets between forms I and II crayfish.
Smooth setae were identified as having a smooth outline with pointed tips and an apical terminal pore (Thomas, 1970). Plumose setae were feather-like, with long setules (or wisps) coming off the shaft in two opposite rows, and contain a smooth pointed tip (Thomas, 1970). After identification, smooth and plumose setae numbers were calculated by dividing the total number of each setae type by the length of each particular chela. Distal setae pockets were those defined as the first 20 pockets (or 10%) of sensory setae on the chelae because the dorsal, distal tip of the chelae is where odorants would be initially received (Hindley and Alexander, 1978; Solon and Cobb, 1980). Additionally, Hindley and Alexander (1978) showed that the number of smooth (simple) setae became sparser proximally. The remaining pockets of sensory setae were classified as proximal. We used a 3-way ANOVA test (on normalized data) with a Tukey post hoc test to examine the independent variable sensory setae quantity (# setae/pocket/chelae length) with respect to the setae type and location on the major chelae (distal and proximal), as well as the quantity between reproductive forms (dependent variables).

Crystal Violet Staining

To evaluate the porosity of putative chemosensory structures on the major chelae of form I male crayfish, a modified technique of crystal violet staining was used (Slifer, 1960; Cate and Derby, 2002a). Major chelae were fixed in 4% PFA in 0.1 M PB for at least 12 hours and rinsed in 0.1 M PBS for 10 minutes the next day. Debris was not removed from the chelae by sonication in order to prevent membrane and setae damage, which may lead to false positives (Thomas, 1970). Chelae were coated with a solution of 0.3% filtered aqueous crystal violet (Becton Dickinson, Maryland) for 15 seconds using a syringe. After two 15 minute rinses in distilled water, the major chelae were dried and cleared in xylene for 15 seconds. Smooth setae were examined for crystal violet uptake using a Leica (M2 FLIII; Leica Microsystems Inc.,
Bannockburn, Illinois) dissecting scope equipped with a Sony® Power HAD camera (New York, New York). Images were captured with Northern Eclipse 6 software (Empix Imaging, Inc., Mississauga, Ontario).

For better resolution and image quality, the thin fibrous plumose setae were photographed using a brightfield microscope. Because of poor contrast between the plumose setae and the chelae exoskeleton, it was necessary to use thick sections of chelae to examine these setae. After crystal violet staining (outlined above), sections through the chelae were made after the cuticle was softened by immersion in Decal® for at least 24 hours (bone decalcifier, Decal Corporation, Tallman, New York). Following this, chelae tissue was cryoprotected, frozen, and sectioned at 50 µm on a transverse plane using a cryostat (Microm). Sections containing plumose setae were analyzed and photographed using an Axioskop 2 FS mot plus microscope (Carl Zeiss Canada Ltd., Toronto, Ontario) equipped with an RGB colour filter (Qimaging, Burnaby, British Columbia) and a QICAM fast cam 1394 (Qimaging).

**Anterograde Tract Tracing**

In order to determine whether DiI (1,1’-dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate) positive neural components were contained within the sensory setae, we used an anterograde labeling method successfully developed for neural staining in crustaceans by Bundy and Paffenhöfer (1993). This study showed that neurons and their extensions into chemosensory setae could be identified using this technique since the exoskeleton of the setae is transparent. The presence of neural innervation along the setal shaft to the pore or distal tip is indicative of chemosensory setae (McIver, 1975; Altner et al. 1983). DiI is a fluorescent lipophilic dye (excitation 549 nm, emission at 565 nm) that travels by lateral diffusion along the lipid membranes of the neurons.
A solution of DiI (Molecular Probes Eugene, Oregon) crystals was made by dissolving 1 mg of DiI crystal in 1 mL of 95% ethanol. The major chelae were removed from crayfish, rinsed in for 15 minutes 0.1M PBS, and immersed in the DiI solution for 30 minutes (adapted from Bundy and Paffenhöfer, 1993). As in the porosity assay, chelae were not sonicated in order to prevent structural damage to the sensory setae (Thomas, 1970) and therefore debris may be present in the area of the setae pockets. After incubation in the DiI solution, chelae were again rinsed with 0.1 M PBS for 15 minutes prior to fixation in 4% PFA. Chelae were then incubated for 1 week in 4% PFA at 37°C in the dark. A whole mount preparation was imaged using a Leica (M2 FLIII) dissecting scope equipped with a Sony® Power HAD camera (New York, New York) and a green fluorescence filter (Chroma 11002v2; Chroma Technology Corp, Rockingham, Vermont). Images were captured with Northern Eclipse 6 software (Empix Imaging, Inc., Mississauga, Ontario).

**Acetylated Tubulin Immunocytochemistry**

As in Belanger et al. (2003), acetylated tubulin (AT) immunocytochemistry was used to label microtubules contained within neural processes of sensory tissue. The antibody has high specificity and has been used as a marker to label neural components since tubulin is the major building block of microtubules contained within the nervous system (Siddiqui et al., 1989). The major chelae of crayfish were removed from the body, cut into smaller pieces (~ 7 mm x 7 mm) in 0.1 M PBS using a scalpel, and fixed in 4% PFA overnight. Subsequently, they were rinsed for 10 minutes in 0.1 M PBS and the cuticle was softened in Decal® for at least 24 hours. Chelae tissue was then washed for 10 minutes in 0.1 M PBS, frozen after cryoprotection using a sucrose gradient, and sectioned (25 µm) using a cryostat. All fluorescence immunocytochemistry was carried out using a protocol established by Belanger et al. (2003). Chelae sections were
immersed for 10 minutes in cold (4°C) 0.1 M PBS, post fixed in cold acetone, and then washed in cold 0.1 M PBS again. Following this procedure, the non-specific staining was blocked by placing the sections in 0.25 % normal horse serum in 0.1 M PBS for 20 minutes. Chelae tissue was then incubated in the primary monoclonal antibody against acetylated tubulin (anti-mouse acetylated tubulin 1:1000 in 0.4% triton X 100 in 0.1 M PBS; Sigma, St. Louis, MO, USA) for 12 hours at 4°C. After incubation, sections were washed 3 times for 10 minutes each in cold 0.1 M PBS. An avidin/biotin blocking kit (Vector Labs, Burlingame, California) was used to prevent nonspecific staining. Avidin was applied for 10 minutes, followed by a 10-minute rinse in 0.1 M PBS, and lastly biotin was applied to the sections for 10 minutes. Following the biotin application, chelae sections were rinsed for 10 minutes in 0.1 M PBS and treated for 2 hours with a biotinylated secondary antibody (1:100; antimouse IgG; Vector Labs, Burlingame, California) at room temperature (~25°C). For final visualization, Alexa 568 conjugated streptavidin (1:100; Molecular Probes, Eugene, Oregon, USA) was applied for 2 hours in the dark at room temperature, washed 3 times for 10 minutes each in 0.1 M PBS, and mounted in Vectashield (Vector Labs, Burlingame, California). Negative controls were prepared similarly, however the primary antibody was not present in the preparation. Confocal microscopy (BioRad MRC 1024, Hercules, California) was used to obtain images.
Results

Sensory Setae Density and Distribution

Scanning electron micrographs of the dorsal surface of the major chelae of both reproductive (form I; Figure 5A) and non-reproductive male crayfish (form II; Figure 5B) show that the dorsal surface of the major chelae is lined with discrete pockets of sensory setae (examples boxed in Figure 5A and B respectively). Representative pockets from the distal portion of the major chelae have been shown to contain up to 35 smooth setae (Figure 5C). Pockets of sensory setae, located proximally on the chelae, contain a mix of both smooth and plumose setae (Figure 5D).

Quantification of Sensory Setae Pockets

After counting the pockets of sensory setae on the dorsal surface of the major chelae, form I males were found to have significantly more pockets of sensory setae than form II males (p < 0.01, t = 3.46, df = 8, N = 10; t-test). Form I males have 57.97 ± 1.03 pockets of sensory setae/chelae length (CL) (mean ± S.E.) and form II males have 48.65 ± 2.49 pockets of sensory setae/CL (Figure 6A). Results of the 3-way ANOVA test showed that sensory setae quantity differed significantly with respect to form (F (1,32) = 6.46, p < 0.05), location on the major chelae (F (1,32) = 165.02, p < 0.0001), and sensory setae type (F (1,32) = 29.58, p < 0.0001).

Quantification and Distribution of Simple Setae

Dense pockets of predominantly smooth setae are found in significantly higher numbers distally on the major chelae of both form I (3.88 ± 0.22 smooth setae per pocket/CL) and form II (2.60 ± 0.29 smooth setae per pocket/CL) male crayfish (p < 0.001; 3-way ANOVA; Figures 5C and 6B). Proximal pockets of sensory setae of form I males contained an average of 0.61 ± 0.05 smooth setae per pocket/CL and form II males had 0.84 ± 0.08 smooth setae per pocket/CL.
Form I males have significantly more smooth setae on the distal part of their major chelae than form II males (p < 0.001; 3-way ANOVA). There was no difference in the number of smooth setae found proximally when form I and form II males were compared (p > 0.05; 3-way ANOVA; Figure 6B). These data reveal that discrete sensory setae pockets and individual smooth setae are more abundant in form I crayfish.

*Quantification and Distribution of Plumose Setae*

No significant difference was observed in the distribution and density of plumose setae either spatially or across reproductive form (p > 0.05; 3-way ANOVA; Figure 6B). Form I males had $1.55 \pm 1.10$ plumose setae per pocket/CL distally and $1.28 \pm 0.09$ plumose setae per pocket/CL proximally. Form II males had $1.56 \pm 0.11$ plumose setae per pocket/CL distally and $1.32 \pm 0.07$ plumose setae per pocket/CL proximally.

*Spatial Comparison of Setae Types on the Major Chelae*

Overall, smooth setae on the major chelae of male crayfish (form I and II) were more abundant distally (4.5:1; distal:proximal). Male crayfish contained over two times as many smooth setae on the distal portion of the major chelae than plumose setae (2.3:1; smooth setae distal:plumose setae distal). Plumose setae were evenly distributed across the major chelae (0.93:1; distal:proximal).

When reproductive forms were compared, form I males contained significantly more (p < 0.05; 3-way ANOVA; Figure 6B) smooth setae located distally than form II males (1.5:1; smooth setae FI: smooth setae FII). However, in the proximal region of the chelae, form I and II male crayfish contained similar amounts of smooth setae (0.73:1; smooth setae FI: smooth setae FII). Plumose setae were found in approximately equal abundance (p > 0.05; 3-way ANOVA;
Figure 6B) when form I and II were compared (1:0.86 and 1:1.03; plumose setae:plumose setae, proximal, for form I and II respectively).

Evaluation of Porosity

High-powered scanning electron micrographs of the distal tips of both smooth and plumose setae reveal that only smooth setae contain a terminal pore-like structure (sensu: Ball and Cowan, 1977; Hindley and Alexander, 1978; Felgenhauer and Abele, 1983; Altner et al., 1983; Jacques, 1989; Garm, 2004) (Figure 7A), while there was no terminal pore present in plumose setae (Figure 7B). A porosity assay utilizing crystal violet confirmed this. A light micrograph of smooth setae demonstrates that after exposure to crystal violet, smooth setae stained darkly in the distal end (arrows in Figure 7C) and the stain could not be removed by rinsing the cuticle. After 30 seconds, the proximal region of the smooth setae was also stained with crystal violet (data not shown). This indicates that the distal portion of each smooth seta may contain a pore that is permeable to water-soluble molecules. In contrast to smooth setae, plumose setae were crystal violet negative after treatment with the dye and clearing (Figure 7D). These results suggest that the distal portion of each smooth seta may allow chemical stimuli from the environment to enter the lymph cavity located within the sensory setae. Plumose setae do not allow water-soluble dyes to enter into the sensory setae.

Anterograde DiI Labeling

Anterograde DiI labeling of putative neural processes within smooth setae of the major chelae occurred after immersion in a DiI solution and subsequent incubation. Liquid DiI diffused through the cuticle of the smooth setae and stained prominent dendrite-like fibers that extended from the base of the smooth setae to the distal tip just beneath the terminal pore region (Figure 8A). The bright fluorescence indicates the presence of neural innervation along the setal
shaft to the distal tip; indicative of chemosensory setae (McIver, 1975; Altner et al., 1983).

Plumose setae did not take up the liquid DiI and dendrite-like structures were not seen along the length of the plumose setae (Figure 8B). After examination of the sensory setae, the exoskeleton of the chelae was dissected. Beneath the pockets of sensory setae, putative nerve fibers that were DiI positive were visualized (Figure 8C). Therefore, DiI labelled the dendrite-like structures found along the length of the smooth setae, subsequently filling putative nerve fibers located beneath the exoskeleton, suggesting that smooth setae are putative chemosensory setae.

*Acetylated Tubulin Immunocytochemistry*

The use of antibodies against AT revealed that sensory setae on the major chelae are innervated with immunoreactive fibers. Sections through the major chelae revealed that putative nerve fibers which are located beneath the exoskeleton and were visualized using confocal micrographs representing collapsed stacks of optical sections. These nerve fibers originated from beneath the chitinous exoskeleton, ran through the exoskeleton into the proximal portion or base of the sensory setae (Figure 9A). In longitudinal sections through smooth setae, AT immunoreactivity is prominent in the putative dendrites, which extend from the base of the sensory setae to the distal tip of the setae just beneath the terminal pore (Figure 9B). AT immunoreactive dendrites were absent in plumose setae (Figure 9C). Taken together, this suggests that smooth setae are innervated along the length of the setae with dendritic structures, while plumose setae were negative for AT immunoreactivity in this region.
Discussion

In this study, we found that form I males had more pockets of sensory setae, as well as more individual smooth setae on their major chelae compared to form II males. We also showed that there is a significant difference in the number and distribution of sensory setae types among reproductive forms; however the types of sensory setae found on the chelae of both forms are similar. We used different morphological techniques to determine the putative sensory role of these setae. Smooth setae were labeled as putative chemoreceptors because these setae contain an apical terminal pore-like structure, are permeable to water soluble dyes, contain dendrites which extend from beneath the exoskeleton, along the length of the setae, to the distal tip or terminal pore region. Plumose setae were identified as putative mechanosensory setae as they did not contain a terminal pore or dendrites located within the shaft of the setae and were not permeable.

Form I males have more smooth setae on their major chelae than form II males. Smooth setae are concentrated at the distal portion of the chelae of both form I and II males. This result is supported by Thomas (1970) who showed that smooth setae are widely distributed, but are particularly well developed on the distal corners of limb segments. Hindley and Alexander (1978) found that distally, the groups of smooth (simple) setae on the chelae of the banana prawn (*Penaesus merguiensis*) lie close together and contain up to 15 setae. They also found that the distribution of the groups became sparser proximally. In *O. rusticus*, distal setae pockets contained dense pockets of predominantly smooth setae, while proximal pockets contained considerably fewer smooth setae, evenly mixed with plumose setae (Figure 5C and D). Because smooth setae are found in higher numbers distally on the chelae of form I males, we speculate that smooth setae may serve some sensory function related to reproduction.
Form I males have more pockets of sensory setae on their major chelae than form II males. We show that when a male moults into the reproductive form they increase the number of pockets of sensory setae found on the chelae. A reduction in sensory pockets on the major chelae in addition to changes in sensory hair abundance and distribution may be responsible for the differences in behavioural responses to female odours (Belanger and Moore, 2006). Also, loss of the chelae or chelae function can affect mating success (Sekkelsten, 1988; Abello et al., 1994; Juanes and Smith, 1995; Keller and Hazlett, 1996). If the ability to discriminate and locate female odours is more important during reproductive than non-reproductive periods, form I males should show an increase in the number of sensory setae and show differences in their distribution along the major chelae. Our findings support this prediction (Figure 6). In addition, distributions of smooth setae may allow the chelae to function as a multi-channel sense organ, providing information about a number of environmental variables (Solon and Cobb, 1980). Plumose setae are found evenly distributed across the surface of the major chelae in both form I and II males. Plumose setae contain setules which function to increase the surface area of sensory appendages (Drach and Jacques, 1980) and may serve an important mechanosensory function (Bender et al., 1984), important to both forms of crayfish.

External morphological features can be diagnostic of the sensory modality of setae found covering the rigid exoskeletons of crustaceans. Many types of setae have a combined chemo-mechanosensory function (Jacques, 1989); however, the presence of a pore and permeability to water-soluble dyes is indicative of chemosensory abilities (McIver, 1975; Mauchline et al., 1977; Altner et al., 1983; Schmidt and Gnatzy, 1984; Cate and Derby, 2002a; Schmidt and Derby, 2005). Smooth setae have an apical terminal pore-like structure and appear to be permeable to external, water-soluble chemicals (Figures 7A and C). This evidence is supported by Hindley
and Alexander (1978), who also found that simple (smooth) setae of banana prawns contained an apical terminal pore and are believed to have chemosensory abilities. Smooth setae readily take-up a crystal violet solution in a straw-like fashion (Figure 7C); crystal violet travels in a distal to proximal path, indicating that the only point of entry is the terminal pore. This is similar to results found in other crustaceans (e.g. Gleeson, 1982; Altner et al., 1983; Tierney et al., 1986; Cate and Derby, 2002a). Contrary to smooth setae, external morphological analysis of plumose setae did not reveal the presence of a terminal pore; they contain a smooth, pointed distal tip as reported previously by Thomas (1970). Along with the absence of a terminal pore, plumose setae were not permeable to crystal violet and therefore may not be able to take-up odours found in their aquatic environment.

After immersion in DiI, fluorescently labeled dendrite-like structures were found inside the setae, along the length of smooth setae. These structures extend from the base of the setae to the distal tip. Bundy and Paffenhöfer (1993) found that DiI intensely labels the chemosensitive aesthetascs of copepods when externally applied. DiI also labeled putative nerve fibers found beneath the exoskeleton and pockets of sensory setae of the chelae. Conversely, plumose setae were not labeled with DiI and are not innervated with dendrite-like structures. This result is supported by the fact that plumose setae have been shown to be non-innervated mechanoreceptors (Bender et al., 1984). Setae, unlabeled using this technique, should be considered putative mechanosensory setae (Bundy and Paffenhöfer, 1993).

Cross-sections through smooth setae contained AT immunoreactive processes that extended from under the exoskeleton to the tip of the sensory setae, just beneath the terminal pore region. Sensory setae dendrites are small and uniform and are packed with microtubules that extend the length of the hair to the pore (Ball and Cowan, 1977; Schmidt and Gnatzy, 1984).
Cross-sections through plumose setae did not reveal the presence of positively labeled fibers and were classified as non-innervated. These staining techniques, anterograde labeling with DiI and AT immunocytochemistry, provide strong evidence indicating that smooth setae contain putative chemosensory dendrites that extend the length of the setae to the terminal pore region. The presence of a pore and dendrites within the sensory setae offer the morphological criteria necessary for determination of chemosensory function (reviewed in McIver, 1975). However, these setae may have other functions since electrophysiological recordings have shown that smooth (simple) setae are highly sensitive bimodal neurons with specific responses to vibration, deflection, bending, and chemical stimuli (Hatt, 1986; Garm et al., 2004).

The results presented in this study suggest that the major chelae of crayfish serve a chemosensory function that is related to the detection of reproductive odours (Belanger and Moore, 2006) and that the chemosensory function of the major chelae is dependent upon the reproductive form of the crayfish. The morphological results suggest that the smooth setae serve a putative chemosensory function and plumose setae are putative mechanoreceptors. It is possible that the smooth setae serve a bimodal function. Conclusions on sensory function are based on the finding that smooth setae have a terminal pore-like structure, are porous to water soluble dyes, and contain labeled dendrites that extend from beneath the exoskeleton to the distal tip of the setae, just below the terminal pore. Conversely, plumose setae were not shown to be porous or innervated. In addition, the number and distribution of sensory setae changes with reproductive state of the crayfish. Since the behavioural detection of reproductive odours change with reproductive form, the underlying mechanism for this behavioural change probably rests with the morphological changes outlined in this paper. Thus, the major chelae have an important function in the perception of female odours during reproduction and may provide additional
chemical feedback necessary for discrimination of female stimuli. Chelae chemoreceptors may in concert with antennular aesthetasc (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992) by providing important feedback information on the location and type of conspecific odour source. Future electrophysiological experiments examining differential responses of form I and II male *O. rusticus* major chelae to female odours will lead to a better understanding of peripheral processing of odorants.
CHAPTER IV.

THE ROLE OF THE MAJOR CHELAE IN THE LOCALIZATION AND SAMPLING OF FEMALE ODOURS BY MALE CRAYFISH (Orconectes rusticus)\textsuperscript{4}

Introduction

Observations of animal behaviour, both terrestrial and marine, have implicated chemical communication as the prime mode of communication in most animal phyla (Kittredge and Takahashi, 1972). Chemical cues are used by animals as a guide to a future action, such as attraction to conspecifics (Hansson, 1994) and many animals rely on chemical cues for mate choice and species recognition (Wyatt, 2003). Behavioural studies indicate that many crustaceans rely on chemical cues for localization of odour sources (Devine and Atema, 1982; Moore et al., 1991; Kraus-Epley and Moore, 2002), discrimination of odours and odour sources (Derby et al., 1989; Adams et al., 2003; Wolf et al., 2004), communication with conspecifics (Atema, 1986; Schneider et al., 2001; Bergman et al., 2003), and identification, localization, and sex recognition for mating purposes (Atema and Engstrom, 1971; Dunham, 1979). Detection of chemical cues in crustaceans occurs via peripheral chemoreceptors located within the sensory setae of cephalothoracic appendages (Laverack, 1988; Derby, 1989).

Several studies have investigated the use of reproductive cues by female crayfish and subsequent behavioural responses by conspecific males (e.g. Ameyaw-Akumfi and Hazlett, 1975; Gaudioso Lacasa and Cabello, 1979; Tierney and Dunham, 1982, 1984; Dunham and Oh, \textsuperscript{4}Submitted for publication as: Belanger, R.M. and P.A. Moore. In review. The role of the major chelae in the localization and sampling of female odors by male crayfish (Orconectes rusticus). Journal of Chemical Ecology.
1992; Bechler et al., 1988; Villanelli and Gherardi, 1998; Acquistapace et al., 2002; Stebbing et al., 2003a,b; Belanger and Moore, 2006). Responses include attraction (Ameyaw-Akumfi and Hazlett, 1975; Gaudioso Lacasa and Cabello, 1979; Bechler et al., 1988; Tierney and Dunham, 1982, 1984; Stebbing et al., 2003a,b), attempted copulation (Gaudioso Lacasa and Cabello, 1979; Villanelli and Gherardi, 1998), decreased aggression (Ameyaw-Akumfi and Hazlett, 1975; Dunham and Oh, 1992; Hazlett, 1985), increased motility (Acquistapace et al., 2002; Stebbing et al., 2003a), increased urine output and current generation (Simon and Moore, 2007), and increased handling of a female odour source (Stebbing et al., 2003a; Belanger and Moore, 2006). Mating cues are believed to be released in the urine of female crayfish (Stebbing et al., 2003a) and output may be increased during reproductive pairings (Simon and Moore, 2007). It is thought that these mating cues are detected by aesthetasc hairs found on the lateral antennule (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992); however, there is a growing body of evidence that suggests that the major chelae may also be important for female odour perception and may provide feedback to the antennules (Belanger and Moore, 2006).

One of the behavioural indicators that suggest the importance of chelae in sex recognition is chelae sampling behaviour in the presence of conspecific odour. Chelae waving behaviour has been described by Thorp and Ammerman (1978) and Itagaki and Thorp (1981) in crayfish (Procambarus clarkii) where they demonstrated that chelae waving increased in response to a conspecific odour. Later, Dunham and Oh (1992) suggested that chelae waves represent potential sex discrimination in this crayfish. Chelae waving behaviours may be similar to antennular flicking (Snow, 1973; Moore et al., 1991; Goldman and Koehl, 2001; Kraus-Epley and Moore, 2002) where the waving may facilitate the movement of chemicals to the microenvironment of
the sensory cells, contained within the sensory setae of the major chelae. However, it is unclear whether chelae sampling is important for localization and discrimination of sex cues or whether crayfish are capable of performing these behaviours without sensory information from the chelae.

The goal of this study was to determine the role of sensory information obtained from the major chelae of male crayfish (O. rusticus) in two important behaviours: localization of female odour source and discrimination of sex. To accomplish this, we examined how male crayfish localize and discriminate female odours and whether these behaviours are altered by lack of sensory information from the chelae. We hypothesized that male crayfish with intact major chelae will be able to localize the source of a conspecific female and will exhibit chelae waving in response to this odour and that these behaviours will be eliminated without chemosensory information from the major chelae.
Materials and Methods

Animals

*Orconectes rusticus* (females and form I males) were collected from the Portage River near Bowling Green State University in Bowling Green, Ohio, U.S.A. Intermoult form I male crayfish used in these experiments were identified by examining their reproductive stylets (Crocker and Barr, 1968). Crayfish were visually and mechanically isolated from each other in a flow-through holding system (each container was 11 cm depth x 17 cm width x 27 cm length). The flow-through tanks were housed in an environmental chamber (23°C, 14:10 hr light dark cycle). Crayfish males were isolated for at least 48 hr before the start of an experiment. Crayfish mass, carapace, and chelae lengths (± S.E.) were measured. Only form I males (16.65 ± 0.65 g; 3.54 ± 0.04 cm carapace length; 3.50 ± 0.07 cm chelae length) with intact appendages (e.g. antennae, lateral and medial antennule filaments, chelae, maxillipeds, and walking legs) were used in this experiment. Reproductive female crayfish (13.42 ± 0.71 g and 3.43 ± 0.05 cm carapace length) were housed in a similar manner prior to collection of odour. Crayfish were fed a diet of rabbit pellets three times per week. Experiments were completed between January and February 2007. All treatments were performed between 0900 hr and 1800 hr.

Conditioned Water Stimulus and Setup

To examine male attraction and chelae sampling behaviours in response to a conspecific odour source, female and male-conditioned water was obtained using a procedure similar to Belanger and Moore (2006). Female-conditioned water was obtained from eight reproductive female *O. rusticus* held individually in clear plastic pots (40 cm x 20 cm x 25 cm) containing 500 ml of aerated dechlorinated water for a 24-hr period. Water from each pot was then collected, combined, and filtered (Whatman® 185 mm #1004185) to remove debris and subsequently used
for behavioural trials. Male-conditioned water was collected in a similar fashion using 8 (form I) males. There was no significant difference in mass or carapace length between the female and male crayfish odour donors used in this experiment ($P = 0.352, t = 0.964, df = 14, N = 16$; t-test). The control for this experiment was dechlorinated water that had been aerated over a 24-hr period in the same style of clear pots, with no crayfish present. The water was then subsequently filtered in a similar manner to the previous conditioned water treatments.

**Experimental Design**

Experiments consisted of a 2 x 3 design with two treatments (intact chelae and blocked chelae) and three odours (female-conditioned water, male-conditioned water, and dechlorinated water (control)). Within each treatment/odour combination, 10 crayfish were tested. Each crayfish was used only once and a total of 60 crayfish (2 treatments $\times$ 3 odours $\times$ 10 crayfish) were used in this study.

**Sensory Blocking Protocol**

The term “intact” refers to a crayfish with the ability to derive sensory information from their major chelae as opposed to “blocked” which refers to crayfish with chemo- and mechanosensory information from their major chelae eliminated. The blocking procedure, similar to Belanger and Moore (2006), was carried out by applying a thin layer of superglue (Duro Quick Gel®) to the dorsal surface from the base to the tip of the major chelae, covering the pockets of sensory setae (Belanger et al., 2008). A drying accelerator (Zip Kicker™) was applied to the superglue with a cotton swab to speed drying time. Intact animals were handled and manipulated in a similar fashion with a similar amount of glue placed at the base of the carapace as a control for the presence of superglue. Chelae of intact animals were washed with tank water using a syringe to simulate the physical stimulation of the chelae of experimental individuals.
during gluing. When the glue was completely dry (~2 min), a spot of liquid correction fluid was applied to the carapace of each individual crayfish to facilitate visualization during trials. Following this, each crayfish was placed in the test arena to acclimate.

**Test Arena**

The flow-through test arena (120 cm long × 21 cm wide × 19 cm deep) contained an inflow and outflow valve and was filled initially with 10.5 l of aerated dechlorinated water (Figure 10). The inflow valve was located in the center of one end of the test arena hence forth referred to as the proximal side. The inflow valve was 4.5 cm above the bottom and was connected via plastic tubing to a flow meter (Manostat Riteflow #2, Manostat, Peaquannock, New Jersey). Attached to the flow meter was a 3 l vat containing the test odorants: female-conditioned water, male-conditioned water, or water (control). After each trial, the test arena and tubing were thoroughly rinsed. Each trial was recorded using a Canon XL-1 digital video camera (overhead camera) mounted above the test arena which was used to monitor large-scale locomotory behaviours and a Sony 3CCD digital video camera (small-scale behaviour camera) for monitoring behaviours that occurred at or near the odour source (Figure 10).

**Experimental Trials**

Blocked and intact male crayfish were placed individually at the distal end (furthest away from the odour source) of the test arena in a cage made of egg crating (8.5 cm wide x 8.5 cm length x 7 cm high). They were then allowed to acclimate to the test arena for 30 min before the start of the experiment. Following the acclimation period, the cage was removed from the test arena and crayfish were allowed to move about the test arena freely. The male’s movements (proximal and distal to the odour source) and behaviours (see Table 2) in the test arena were recorded for at least 15 min (range 15 - 17 min) before the introduction of the treatment odour
(pre-odor delivery period). Once the crayfish returned to the distal area of the arena (the area the crayfish was in when trial was initiated) the test odour was introduced (flow rate 50 ml/min) and movements and behaviours were recorded for another 15 min (post-odor delivery period) (procedure modified from Thorp and Ammerman, 1978).

**Data Collection and Analysis**

Videos obtained from the overhead and small-scale behaviour cameras were analyzed using a JVC MiniDV/S-VHS Dual Deck VCR (model # HR-DVS3U) and a Sony Trinitron Color Video Monitor (model # PVM-1315Q). Large scale locomotory behaviours were quantified in relation to the position of the odour source. Time spent in the proximal area (0 - 60 cm from source) versus distal (60 -120 cm from source) was calculated from the overhead camera view. Proximal and distal times were measured pre- and post-odour delivery. Because of slight differences in trial lengths (due to the need to start trials when animals were in the distal section), these times were then transformed into a percentage of the total time of the trial. In order to measure odour stimulated behaviour above normal baseline activity, the percentage of time spent in each area during the pre-odor period was subtracted from the time spent in the proximal and distal sections during the odour stimulation period (Thorp and Ammerman, 1978; Schmidt and Derby, 2005). Time (sec) at the odour source was recorded when the major chelae of the male crayfish came within 3 cm of the odour source and were positioned with the chelae facing the odour source (Tierney and Dunham, 1982, 1984).

Small-scale behaviours performed within close proximity of the odour source were quantified from the small-scale camera. These behaviours included: chelae waving, chelae opening and closing, chelae open, chelae odour source contact, and meral spread (for definitions of each behaviour; see Table 2). Again, to measure odour stimulated behaviour above normal
baseline activity, the time spent performing each behaviour during the pre-odour period was subtracted from the time spent performing behaviours post-odour delivery.

*Statistical Analysis*

All proportions were transformed using the square-root arcsine transformation (Zar, 1999). A three-way ANOVA test (with odour type, treatment group and test arena location as factors) with a Tukey-HSD post-hoc test was used to investigate differences in the proportion of time spent in each area of the test arena. A two-way ANOVA (with odour type and treatment group as factors) with a Tukey-HSD post-hoc test was used to investigate differences in time spent at the odour source. Proportion of time performing behaviours (time doing a specific behaviour/ total time doing all behaviours for a particular treatment) at the odour source was analyzed using a multiple comparisons for proportions contingency table ($q_{0.05,\infty,4} = 5.301$) that allows for testing analogous to the Tukey or Student–Newman–Keuls tests (Zar, 1999). Significant results are represented by giving $q_{0.05,\infty,4} > 5.301$ from the multiple comparisons test, which is equivalent to $P < 0.05$. 
Results

Time in the Proximal and Distal Sections of the Test Arena

Results of the three-way ANOVA test showed that crayfish spend significantly different percentages of time in the different sections of the test arena depending upon treatment and odour type \( (F_{1,108} = 6.41, P < 0.05) \). Also, there was a significant interaction between odour types and sections of the test arena \( (F_{2,108} = 8.74, P < 0.001) \).

(1) Female-Conditioned Water Responses: Intact male crayfish spent a significantly higher percentage of time (mean ± S.E.) in the proximal region of the test arena than in the distal section when a female odour was present \( (45.4 ± 7.7 \% ; P < 0.05) \). There was a \( 39.4 ± 10.8 \% \) increase in the amount of time spent in the proximal versus distal section of the test area by male crayfish with blocked chelae; however, there was no significant difference between the time spent in proximal and distal section \( (P > 0.05; \text{Figure 11}) \).

(2) Male-Conditioned Water Responses: When both intact and blocked males were exposed to conspecific male-conditioned water, there was no significant increase in amount of time spent in the proximal compared to distal sections of the test arena. There was a \( 14.8 ± 6.7 \% \) increase for intact males and a \( 22.6 ± 9.2 \% \) increase for blocked males \( (P > 0.05; \text{Figure 11}) \).

(3) Water (Control) Responses: There was no significant increase in the amount of time spent in the proximal versus distal sections for intact males exposed to water \( (10.0 ± 8.5 \%, P > 0.05; \text{Figure 11}) \). Likewise, there was no increase in the percent time spent in the proximal versus distal section of the test arena for male crayfish with blocked chelae \( (14.6 ± 4.5 \%, P > 0.05; \text{Figure 11}) \).
**Time at Odour Source**

Intact male crayfish spent significantly more time ($102.0 \pm 24.5$ sec) at the female-conditioned water odour source than both intact ($24.2 \pm 9.2$ sec) and blocked ($17.0 \pm 15.3$ sec) males presented with male-conditioned water ($P = 0.04$ and $0.02$, respectively) and intact ($7.0 \pm 6.0$ sec) and blocked ($4.3 \pm 1.8$ sec) males presented with water ($P = 0.006$ and $0.004$, respectively, Figure 12). There was no significant difference in the amount of time spent at a female-conditioned water odour source for intact ($102.0 \pm 24.5$ sec) and blocked ($71.3 \pm 31.3$ sec) male crayfish ($F_{(1,54)} = 11.49$, $P = 0.82$; two-way ANOVA, Figure 12).

**Chelae Sampling Behaviours**

In order to determine if crayfish had a different behavioural repertoire while at the odour source, we compared the proportion of time spent performing behaviours using a multiple comparisons for proportions contingency table (Table 3). Results of this test indicate that male crayfish with intact major chelae spent a significantly higher proportion of their time chelae waving at a female odour source than all other treatments ($61 \%$, $P < 0.05$). Conversely, blocked male crayfish presented with female odour and intact male crayfish presented with male odour spent more their time ($74 \%$) opening and closing their chelae, instead of waving. When presented with male odours, intact male crayfish spent more time opening and closing their chelae ($94 \%$, $P < 0.05$) and blocked males spent a greater percentage of time ($42 \%$, $P < 0.05$) in contact with the odour source when compared to other treatments and odours. There was no difference in the amount of time male crayfish spent performing meral spreads in response to all odours presented ($P > 0.05$). Overall, there were no recorded behaviours by male crayfish (intact or blocked) in response to water.
Discussion

This study demonstrates that reproductive (form I) male *Orconectes rusticus* with intact major chelae can discriminate, locate, and are attracted to a reproductive female odour source. Form I male crayfish spent more time in the area of the test arena closest to the odour source, spent more time at the odour source, and demonstrated more chemosensory sampling behaviours when presented with female odours when compared to intact males presented with male odours or water control (Figure 11). After the major chelae were blocked from receiving sensory information, these behavioural findings disappear. Male crayfish with blocked major chelae can no longer localize a female odour source and did not perform chemosensory sampling behaviours. Also, intact male crayfish demonstrated increased chelae waving behaviours when they were positioned in front of a female odour source when compared to blocked male crayfish. Taken together, results from this study strongly suggest that the major chelae of form I males are important chemosensory appendages that play a role in attraction, localization, and discrimination of conspecific female odours.

Previous studies have demonstrated that male crayfish are attracted to and localize conspecific female odours, e.g. *Austropotamobius pallipes* (Gaudioso Lacasa and Cabello, 1979), *O. propinquus* (Tierney and Dunham, 1982, 1984), *O. rusticus* (Belanger and Moore, 2006), *Pacifastacus leniusculus* (Stebbing et al., 2003a,b), *Procambarus clarkii*, and *P. acutus* (Bechler et al., 1988). In this study, we found that form I male *O. rusticus* with intact major chelae spent more time in the area proximal to the odour source than the distal section of the test arena (Figure 11). This result is supported by previous work done by Bechler et al. (1988) where they showed that male *P. clarkii* and *P. acutus* displayed a preference for female conspecific odour in a Y-maze. Also, Stebbing et al. (2003a) found that reproductive male crayfish (*P.*
*leniusculus*) were attracted to and exhibited increased levels of activity and handling behaviour in response to a reproductive female odour source. Once the major chelae of form I males were sensory blocked, there is no difference in the amount of time spent in the proximal and distal sections of the test arena. This finding was previously demonstrated by Belanger and Moore (2006) who showed that once the chelae of form I male crayfish were sensory blocked they behaved similarly to non-reproductive (form II) males where there was no response to female odours. Also, form I male *O. rusticus*, with either intact or blocked chelae, showed no preference for any area of the test arena when they were presented with conspecific male odour or water (Figure 11). This result had been previously demonstrated by Thorp and Ammerman (1978), where they showed that when male *P. acutus acutus* were exposed to an odour source of conspecific males housed separately, there was no attraction to the source or aggressive responses. These results clearly indicate that the major chelae of form I male crayfish contain important sensory structures used for the discrimination and localization of conspecific female odours.

Behavioural recognition of sex and reproductive status has been demonstrated in several crayfish species exposed to conspecific conditioned water (reviewed in Dunham, 1988; Bechler, 1995). In this study, we show that form I male *O. rusticus* with intact sensory abilities are attracted to, localize, and discriminate a conspecific female odour source from that of conspecific males and water. Similarly, male *O. virilis* and *O. propinquus* were attracted to and localized a conspecific female odour source (Tierney and Dunham, 1982, 1984). However, once form I male *O. rusticus* were sensory blocked, there is no difference in the time spent at the female odour source when compared to male odour and water (Figure 12). We have previously demonstrated that once form I male crayfish are sensory blocked, they are unable to discriminate female odour
from male odour or water (Belanger and Moore, 2006). This indicates that cues, released by reproductive female crayfish into the aquatic environment, are important for form I male *O. rusticus*, allowing males to be able to locate females for mating.

Although there was no difference in the amount of time intact and blocked males spent at the female odour source, only male crayfish with intact sensory abilities displayed chelae waving behaviours when they were positioned in front of a female odour source (Figure 12). Previously, *P. clarkii* have been shown to perform chelae waving behaviours in the presence of conspecific odour (Itagaki and Thorp, 1981) and it has been suggested that chelae waving may be a sampling behaviour that is used for sex discrimination (Dunham and Oh, 1992). We believe that chelae waving behaviour is a chemosensory sampling event, for three reasons: chelae have been previously shown to have chemoreceptive properties (Bauer and Hatt, 1980; Hatt and Bauer, 1980; Altner et al., 1983), the major chelae are important for discrimination of female odours (Belanger and Moore, 2006), and crayfish exhibit movements of their chelae similar to other appendages (e.g., antennule flicking), which have been shown to be associated with chemosensory sampling (Snow, 1973; Moore et al., 1991; Goldman and Koehl, 2001; Kraus-Epley and Moore, 2002). Therefore, chelae waving may aid in the transport of conspecific odours to the chelae sensory cells and thus male crayfish may employ chelae waving behaviours in order to enhance sensory perception of female odours.

Generally, male crayfish are attracted to female odours [e.g. *A. pallipes* (Gaudioso Lacasa and Cabello, 1979); *O. propinquus* (Tierney and Dunham, 1982, 1984); *P. leniusculus* (Stebbing et al., 2003a,b); *P. clarkii* and *P. zonangularis* (Bechler et al., 1988)]. However, males are only attracted to females that are sexually receptive (Gaudioso Lacasa and Cabello, 1979; Villanelli and Gherardi, 1998) and exhibit increased motility and handing behaviour in response to a
reproductive female odour source (Stebbing et al., 2003a). Also, there is evidence that
demonstrates that form II male *O. rusticus* are not attracted to conditioned water from
reproductive females (Belanger and Moore, 2006) and form II males do not display mating
behaviours with either reproductive or non-reproductive female conspecifics (Simon and Moore,
2007). This suggests that form I male crayfish must therefore recognize and use chemical cues,
released from sexually receptive female conspecifics, for discrimination and localization of
mates. Sensory blocking the chelae of reproductive male *O. rusticus* eliminates this ability,
making their suite of behaviours similar to those of non-reproductive males (Belanger and
Moore, 2006). Given the importance of the major chelae for discrimination and localization,
chelae waving behaviour may also be an important sampling behaviour used for mating and mate
recognition. If so, removing the ability to sense female odours with their chelae, utilizing sensory
blocking, should inhibit the ability of male crayfish to perform behaviours essential for
reproduction.

The results from this and other studies indicate that the major chelae of the crayfish are
important chemosensory appendages mediating crayfish behaviours in regard to recognition of
sex and/or mating odours. This is evident as a suite of behaviours occurs when a form I male
crayfish comes into contact with a reproductive female odour source. First, male crayfish use
sensory information, obtained from their major chelae, to discriminate a female odour source
from other odours (Belanger and Moore, 2006; this study). Secondly, they localize the female
odour source and begin sampling the odour using chelae waving (this study), and lastly grab and
handle the female odour source with their major chelae (Stebbing et al., 2003a; Belanger and
Moore, 2006). Congruent with this theory, is the finding that form I male crayfish have more
robust chelae and a higher number of chemosensory setae on their chelae compared to the chelae
of form II males (Belanger et al., 2008). It is possible that chemoreceptors, found on the major chelae, may work with other appendages (e.g. antennular aesthetasc Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992) by providing important feedback information on the sequence of behavioural decisions necessary for mate choice and localization. Finally, several studies have demonstrated the use of reproductive odours by female crayfish to attract conspecific males (reviewed in Dunham, 1988; Bechler, 1995). Overall, it can be concluded that the major chelae are not only necessary for the mechanical manipulation of females during mating interactions, but may also play an important chemosensory role in discrimination, localization, and even selection of reproductively receptive females.
CHAPTER V. SUMMARY AND GENERAL CONCLUSIONS

This dissertation uses an interdisciplinary approach to investigate the use of the major chelae by male crayfish (*Orconectes rusticus*) for discrimination and localization of female odours. This work demonstrates three major findings about the chemosensory abilities and morphology of crayfish major chelae and ultimately leads to a better understanding of the importance of the major chelae for mating and reproduction. First, reproductive (form I) male crayfish use sensory structures, found on their major chelae, for perception of female odours. Second, form I crayfish had more pockets of sensory setae and individual smooth (simple) chemosensory setae on their major chelae when compared to non-reproductive (form II) males. Third, the major chelae are important sensory structures needed for the discrimination and localization of female odours. Also, it was found that male crayfish may employ chelae waving behaviours in order to enhance sensory perception of female odours.

In chapter 2, a behavioural bioassay was used to investigate whether male form I and form II crayfish use their major chelae to detect reproductive female odours. The behavioural reactions of form I and form II males were recorded and analyzed in response to four different odour treatments: reproductive female-conditioned water, male-conditioned water, filtered fish homogenate (food odour; positive control), and water (negative control) delivered from an air stone. In addition to this, all males were under two sensory conditions: intact or blocked chelae. Normalized odour source handling time was measured, along with time spent handling the odour source between differing groups of sensory appendages: major chelae/first walking legs and maxillipeds/first walking legs. Results of this study indicate that both form I and II, intact and blocked, male crayfish significantly handled the odour source after a food stimulus was introduced. However, only intact form I males handled the odour source significantly when a
reproductive female odour was delivered (Chapter 2; Figure 3). Sensory-blocking of the chelae of form I males eliminated this difference. Intact and blocked form II males showed no differences when presented with reproductive female odours. Also, intact form I male crayfish spent more time handling the reproductive female-conditioned water source with their major chelae/first walking legs than those crayfish exposed to food odours (Chapter 2; Figure 4). Overall, these results strongly suggest that the major chelae contain necessary chemosensory structures needed for female odour source recognition.

With the knowledge that form I male crayfish require sensory information, obtained from their major chelae, for discrimination of conspecific female odours, an analysis of the sensory setae found on the chelae of both form I and II male crayfish was performed. The goal of Chapter 3 was to determine the distribution and putative sensory function of smooth and plumose setae, found on the major chelae. Specifically, the distribution of setae between reproductive forms was quantified, as well as putative sensory functions, based on morphological characteristics, for both smooth and plumose setae. To accomplish these goals, scanning electron microscopy, a porosity assay using crystal violet, anterograde labeling using DiI, and acetylated tubulin (AT) immunocytochemistry were used. It was determined that that form I crayfish have significantly more pockets of sensory setae and individual smooth setae on their major chelae when compared to form II males (Chapter 3; Figure 5 and 6). Scanning electron microscopy revealed a terminal apical pore-like structure in smooth setae that was absent in plumose setae (Chapter 3; Figure 7). Congruent with this finding, smooth setae absorbed crystal violet stain and DiI which labeled putative neural fibers (Chapter 3; Figure 7 and 8). Conversely, plumose setae did not show any crystal violet or DiI staining. Furthermore, smooth setae contain fiber-like processes that are AT-immunoreactive and are located below the chitinous exoskeleton
of the chelae. These fibers extend from the base of the smooth setae to the pore-like structure located at the distal tip. AT-immunoreactive fibers were not present in plumose setae (Chapter 3; Figure 9). These results imply that smooth setae on the major chelae are putative chemoreceptors and plumose setae may serve a mechanosensory function. Coupled with previous behavioural studies (Chapter 2), these results suggest that a dimorphism in the major chelae, found between male reproductive forms, could enhance sensory perception during reproductive behaviours.

After completing chapters 2 and 3, it was clear that increases in chemosensory setae, found on the major chelae of form I males, may be responsible for the increase in handling response to conspecific female odours. However, it was unclear whether the major chelae were used solely for discrimination or if the chelae were important for localization of female odour sources. Therefore, in chapter 4, the role of sensory information from the major chelae in the localization and discrimination of different conspecific odours was examined. Behavioural reactions of form I male crayfish were recorded to three different odour treatments: reproductive female-conditioned water, reproductive male-conditioned water, and water delivered from one end of a test arena. Also, all male crayfish either had their chelae intact or blocked from receiving sensory signals. Locomotory behaviours, as well as chemosensory sampling behaviours were recorded in response to odours and chelae treatments. In addition, specific behaviours performed at the source of the odour were quantified. Large scale locomotory behaviours demonstrated that male crayfish with intact major chelae spent more time closer to the odour source when the reproductive female odour was delivered, whereas crayfish with blocked chelae showed no preference. There was no significant response to male-conditioned water or control water (Chapter 4, Figure 11). Also, male crayfish demonstrated differences in
chela waving and other local behaviours in response to odour and chela treatment (Chapter 4, Table 3). These results suggest that the major chelae are important sensory structures needed for the localization and discrimination of female odours and may be important for reproductive behaviours such as mate recognition.

Previous studies have found that the antennular aesthetasc hairs of male crayfish are used for the perception of female odours (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992). These studies showed that when the lateral antennules were removed from crayfish (*O. propinquus* and *Procambarus clarkii*), response to a female odour source decreased significantly (Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992). However, a more recent study by Corotto et al. (1999) in crayfish (*P. clarkii*) found that the localization of females by male crayfish and timing of reproductive activities were not impacted by the removal of the lateral antennules. This dissertation shows that once a male mouls from form II to form I, the number of chemosensory hairs found on the chelae increases (Chapter 3). This correlates with an increase in the response to female odours (Chapter 2), suggesting that chelae chemoreceptors may play an important role in providing additional chemical feedback necessary for discrimination and localization of a female odour source.

To date, research on the major chelae has focused on their importance for the mechanical manipulation of females during mating activities (Stein, 1976; Stein et al., 1977; Sneddon, 1990; Keller and Hazlett, 1996). It has also been shown that form I male crayfish have more robust major chelae in relation to their body size than form II males (Crocker and Barr, 1968; Stein, 1976) and male reproductive success has been positively correlated with chelae length (Stein, 1976). Given this, it has been concluded that the most important purpose of the major chelae is
their use in reproductive activities (Stein, 1976). It has been shown that chelae loss can negatively influence male mating success in crustaceans and that mating is unsuccessful in shore crabs (*Carcinus maenas*) if one or both of the major chelae are not functional (Sekkelton, 1988; Abello et al., 1994). Abello et al. (1994) found that the proportion of male crabs with missing chelae found in mating pairs, both in precopula and copula was much lower than that found in the adult unpaired population. Also, male crayfish (*O. propinquus*) succeeded in copulating with females only when they had use of their chelae (Keller and Hazlett, 1996). Taken together with results obtained from this dissertation, it can be concluded that crayfish lacking one or both major chelae would be disadvantaged as they can not mechanically manipulate their mates or sense them chemically.

Data from this dissertation shows that form II males were not attracted to female odours and that once the chelae of form I males were sensory blocked, their response to female odours became similar to that of form II males. There are several behavioural studies which suggest that there is a difference in the way reproductive and non-reproductive males perceive and respond to a reproductive female odour source (See Table 1 from Belanger et al., 2007; Corkum and Belanger, 2007). With this, it is known that crayfish males are only attracted to females that are sexually receptive (Gaudioso Lacasa and Cabello, 1979; Villanelli and Gherardi, 1998) and that form II males do not display mating behaviours with either reproductive or non-reproductive female conspecifics (Simon and Moore, 2007). It is believed that behavioural differences that occur during the reproductive season likely result when hormones, which may be upregulated at sexual maturity or during the reproductive season, change the expression of chemoreceptors (Alekseyenko et al., 2006). Hormonal changes may also lead to morphological changes associated with increases in odorant receptors at the onset of sexual maturity (Schreibman et al.,
These findings are congruent with the data obtained in Chapter 3 which demonstrates that when form II males moult to form I, they add chemosensory setae and subsequently respond to female odours (Chapter 2). This suggests that changes that occur at the onset of the crayfish reproductive season, specifically increases in the number of chelae chemosensory setae, leads to heightened perception of female odours. With this, chelae waving behaviour may also be an important sampling behaviour used for mating and mate recognition.

Overall, results from this dissertation indicate that the major chelae of male crayfish are important chemosensory appendages mediating crayfish behaviours in regard to recognition of sex and/or mating odours. This is evident as a suite of behaviours occurs when a form I male crayfish comes into contact with a reproductive female odour source. First, male crayfish use sensory information, obtained from their major chelae, to discriminate a female odour source from other odours (Chapter 2 and 4). Secondly, they localize the female odour source and begin sampling the odour using chelae waving (Chapter 4), and lastly grab and handle the female odour source with their major chelae (Stebbing et al., 2003a; Chapter 2). Congruent with this theory, is the finding that form I male crayfish have more robust chelae and a higher number of chemosensory setae on their chelae compared to the chelae of form II males (Chapter 3).

Without sensory feedback from the major chelae, male crayfish do not localize, discriminate, or grab a female odour source. It is also possible that chemoreceptors, found on the major chelae, may work with other appendages (e.g. antennular aesthetascs Ameyaw-Akumfi and Hazlett, 1975; Tierney et al., 1984; Dunham and Oh, 1992) by providing important feedback information on the sequence of behavioural decisions necessary for mate choice and localization. The major chelae are thus more than just a mechanical tool used to manipulate females for mating, they are an essential chemosensory appendage used for the discrimination, localization, and even
selection of reproductively receptive females. It is thus believed that missing or injured major chelae would lead to a decrease in mate success and reproductive fitness.

**Future Directions**

This dissertation demonstrates that the major chelae of male crayfish are important chemosensory appendages needed for discrimination and localization of female odours. Future electrophysiological experiments, examining differential responses of form I and II male *O. rusticus* major chelae afferents, to female odours would lead to a better understanding of peripheral processing of odorants. Coupled with electrophysiology, anterograde transneuronal tracing using neurotropic alpha-herpesviruses can be used for investigating the structural organization of multisynaptic pathways or circuits of several neurons (Oztas, 2003), including those of the major chelae. This would allow us to determine where chemoreceptors of the major chelae project in the brain and would provide insight on how the brain processes and integrates chemosensory information from multiple sensory appendages. Subsequently, we would be able to determine how chemosensory information, obtained from the chelae, relates to that obtained from the antennules. Also, behavioural experiments where both antennules and major chelae are blocked would allow us to determine the contribution of each of these sensory appendages to chemoreception of female odours. Along with this, using reproductive female crayfish, in place of female odour, would corroborate localization, discrimination, and sampling behaviours recorded in response to female odour.
LITERATURE CITED


FIGURE 1. Diagrammatic representation of a cross-section through both chemosensory (left) and mechanosensory setae (right). Chemosensory setae contain a terminal pore or spongy cuticle and chemosensory dendrites that extend along the length of the setae, reaching the pore or distal tip. Also, chemosensory setae typically lack setules. Mechanosensory setae are mounted on a flexible membrane and have an abundant amount of setules. Dendrites do not extend into the setae, but are located at the base of these setae and are attached to one side of the setae or the other. Figure modified from Schmidt and Gnatzy (1984).
FIGURE 2. A. Scanning electron micrograph depicting the dorsal surface of the major chelae of a form I male crayfish containing sensory hair pockets (square). Scale bar in A is 5.6 mm. B. A sensory hair pocket (inset from A) found on the major chelae containing both simple (s) and plumose setae (p). Scale bar in B is 40 µm.
FIGURE 3. A comparison of normalized time in seconds (sec) spent handling an odour source (mean ± SE) for form I (FI) and form II (FII) males that were intact and sensory blocked (N = 20 per treatment) with respect to their major chelae. Significant differences (p < 0.0005) between treatments were denoted as a and b. Shaded bars signify crayfish that were intact while white bars represent crayfish with sensory-blocked major chelae. A. Intact FI males spent significantly more time handling the female-conditioned water source than sensory-blocked FI individuals and both intact and blocked FII males. B. Conspecific male odours did not elicit a significant odour source handling response for both intact and blocked, FI and FII males. C. Fish odour source handling did not vary across all FI and FII treatments (intact and blocked) and were significantly greater than responses by form I blocked and form II intact and blocked males to female odours as well as, all responses to male odour and water. D. When FI and FII (intact and blocked) males were exposed to water, there were no significant increases in odour source handling time.
FIGURE 4. An examination of sensory appendage use for female and food odours for males that were intact or sensory-blocked with respect to their major chelae (N = 20 per treatment). This figure includes normalized handling time (mean in seconds (sec) ± SE) from intact form I males to food odour (IFIF), blocked form I males to food odour (BFIF), intact form II males to food odour (IFIIF), blocked form II males to food odour (BFIIF), and intact form I males to female odour (IFIFe). A. Intact form I male crayfish handled a female-conditioned water odour source significantly more with their chelae/walking legs then male crayfish (FI and FII) exposed to food odours. Significant differences (p = 0.008) between treatments were denoted as a and b. B. An increase in maxilliped/walking leg appendage use was observed for IFIF and BFIF treatments, as well as IFIIF and BFIIF treatments when compared to IFIFe.
FIGURE 5. Scanning electron micrographs showing the dorsal surface of the major chelae of form I (A) and form II (B) male crayfish. The major chelae of crayfish have several pockets of sensory setae containing both smooth and plumose setae (boxed in A and B). A magnified representation of a pocket of sensory setae from the distal portion of major chelae (similar to the pocket highlighted in A), which contains smooth setae (s) in dense amounts (C). Setal pockets containing both smooth and plumose setae (p) are located in the proximal regions (similar to the pocket highlighted in B) of the dorsal surface of the major chelae (D). Scale bars: A, 350 µm; B, 350 µm; C, 100 µm; D, 100 µm.
FIGURE 6. Quantification and distribution of pockets of sensory setae and distribution of sensory setae on the major chelae (mean ± S.E.), corrected for chelae length (CL). Form I males have significantly more pockets of sensory setae on the dorsal surface of their chelae when compared to form II male crayfish (A). Asterisk indicate significant differences. Also, there is a difference in sensory setae distribution. Form I and form II male crayfish have statistically more smooth setae located distally on their major chelae (B). Bars filled with a checkered pattern denote sensory setae located on the distal part of the major chelae, while white bars represent those found proximally for both smooth and plumose setae. Significant differences (p < 0.05) between setae numbers were denoted as a, b, c, and d.
FIGURE 7. Scanning electron micrographs of the distal tips of smooth (A) and plumose (B) setae. A close up of the distal tip of a smooth setae (A) reveals that each seta contains a terminal pore-like structure (arrow). No pore structure is visualized at the tip of the plumose setae (B). Congruent with this finding, a porosity assay demonstrates that smooth setae absorb and retain crystal violet dye (arrows in C) and are crystal violet positive (CV+) when visualized using a whole mount preparation. Sections through the chelae reveal that plumose setae are crystal violet negative (CV-, circled in D). Scale bars: A, 4 µm; B, 5 µm; C, 100 µm; D, 100 µm.
FIGURE 8. Whole mount anterograde Dil labeling of setae found in pockets of sensory setae on the major chelae. Smooth setae are Dil positive and are stained along the length of the sensory setae (A), while plumose setae were Dil negative (B). The exoskeleton of the chelae was dissected and beneath the pockets of sensory setae are putative nerve fibers, which were also Dil positive (arrow in C). Scale bars: A, 100 µm. B, 70 µm; C, 100 µm.
FIGURE 9. Collapsed stacks of confocal micrographs representing acetylated tubulin (AT) immunoreactivity in the sensory setae of the major chelae. Smooth setae contain dendrite-like structures (arrow in A) that are AT immunoreactive and are located below the chitinous exoskeleton of the chelae (A). These dendrites extend from the base of the smooth setae (arrow in A) to the tip (arrow in B). Plumose setae do not contain AT immunoreactive dendrites (C). Scale bars: A, 100 µm. B, 8 µm. C, 70 µm.
FIGURE 10. Schematic of the experimental test arena setup used for examining odour source recognition and sampling behaviours in male crayfish. Chemical stimuli, female-conditioned water, male-conditioned water, or water, were gravity-fed into the experimental test arena. Flow rates were controlled by a flow meter. Two cameras were positioned above the test arena in order to observe large-scale and small-scale behaviours of the crayfish. The overhead camera was used to examine locomotory responses exhibited by each crayfish. The small-scale behaviour camera was focused at the odour source and was used to determine chelae sampling and other localized behaviours.
FIGURE 11. Percent increase (± S.E.) in normalized time spent in proximal versus distal section of the test arena. Values were normalized by subtracting time spent in a section pre-odour delivery from this spent in a section post-odour delivery. Percentage values represent the increased time crayfish spent in the proximal versus distal section upon odour stimulation. Thus, a value of 0% would indicate no change in the time spent in the proximal versus distal section, whereas a value of 100% would indicate a doubling of time spent in the proximal versus distal section. Solid bars denote crayfish with intact major chelae and open bars represent crayfish with blocked chelae. F = female-conditioned water, M = male-conditioned water, and W = water control. * represents that there was a significant difference (P < 0.05; three-way ANOVA with Tukey-HSD post-hoc test) in the time spent in the proximal versus distal section of the test arena.
FIGURE 12. Time (sec ± S.E.) spent at the odour source for intact (solid bars) and blocked (open bars) male crayfish in response to female-conditioned water (F), male-conditioned water (M) and water control (W). Significant differences (P < 0.05; two-way ANOVA with Tukey-HSD post-hoc test) between time spent at the odour source are denoted as a and b.
Table 1. A summary of conspecific chemical communication in crayfish modified from Corkum and Belanger (2007). The taxonomic classification scheme follows Taylor (2002). Genus and species names are listed alphabetically within each family.

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Genus Species</th>
<th>Sex</th>
<th>Test Substance</th>
<th>Behavioural Response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decapoda</td>
<td>Astacidae</td>
<td>Astroptamobius pallipes</td>
<td>M</td>
<td>Conspecific female</td>
<td>Males passed through five stages of attraction to females, with the peak occurring when reproductive females were most receptive to copulation.</td>
<td>Gaudioso Lacasa and Cabello, 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>Receptive and non-receptive females</td>
<td>Males attempted copulation with the receptive females only.</td>
<td>Villanelli and Gherardi, 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>Female conditioned water plus a visual stimulus</td>
<td>Males required both chemical and visual stimuli in order to obtain a behavioural response. Males spent more time moving and less time hiding in a shelter.</td>
<td>Acquistapace et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Cambaridae</td>
<td>Pacifastacus leniusculus</td>
<td>M</td>
<td>Mature female conditioned water</td>
<td>Males exhibited increased levels of motility and handling behaviour.</td>
<td>Stebbing et al., 2003a</td>
</tr>
<tr>
<td></td>
<td>Orconectes propinquus</td>
<td></td>
<td>M</td>
<td>Female conditioned water</td>
<td>Reproductive males with intact major chelae spent more time handling the female odour source than those that non-reproductive males. Reproductive males with blocked chelae.</td>
<td>Belanger and Moore, 2006</td>
</tr>
<tr>
<td>Orconectes rusticus</td>
<td></td>
<td>M</td>
<td>Female conditioned water</td>
<td>Males spent significantly more time within 3 cm of a female odour source.</td>
<td>Tierney and Dunham 1982, 1984</td>
<td></td>
</tr>
<tr>
<td>Orconectes virilis</td>
<td></td>
<td>M</td>
<td>Female conditioned water</td>
<td>Males spent significantly more time within 3 cm of the stimulus inflow hole when conspecific female conditioned water was released.</td>
<td>Tierney and Dunham 1982, 1984</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Procambarus acutus</td>
<td></td>
<td>M</td>
<td>Female conditioned water</td>
<td>A submissive response to cues released by the opposite sex.</td>
<td>Hazlett, 1985</td>
</tr>
<tr>
<td>Procambarus clarkii</td>
<td></td>
<td>M</td>
<td>Females located in perforated test containers and female conditioned water</td>
<td>Males showed submissive behaviours when females were in the test containers and when female conditioned water was introduced. Males showed submissive behaviours when female conditioned water was introduced.</td>
<td>Ameyaw-Akumfi and Hazlett, 1975</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>Conspecific female</td>
<td>Males spent more time in the area of receptive females.</td>
<td>Gaudioso Lacasa and Cabello, 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>Female conditioned water</td>
<td>Displayed a preference for female conspecific odour in a Y-maze.</td>
<td>Bechler et al., 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>Female conditioned water</td>
<td>Less aggressive postures were visualized when males presented with female stimulus water.</td>
<td>Dunham and Oh, 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F</td>
<td>Male conditioned water</td>
<td>Intact females displayed significantly more aggressive behaviours in the presence of stimulus water from the same sex than from males.</td>
<td>Dunham and Oh, 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>Conspecific female</td>
<td>Male crayfish win more bouts and evict more non-reproductive females from their shelter than reproductive females.</td>
<td>Figler et al., 2005</td>
</tr>
</tbody>
</table>
Table 2. Behavioural responses recorded in response to a conspecific conditioned water odour source (modified from Thorp and Ammerman, 1978; Itagaki and Thorp, 1981).

<table>
<thead>
<tr>
<th>No behaviours present</th>
<th>Crayfish positioned in front of the odour source with no chelae movement or contact.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chela(e) waving</td>
<td>Moving the right and/or left chela back and forth in front of the body horizontally in the water column, perpendicular to the substratum.</td>
</tr>
<tr>
<td>Chelae opening and closing</td>
<td>Pincers of both chelae open and closing.</td>
</tr>
<tr>
<td>Chelae open</td>
<td>One or both chelae held apart but not raised from the substratum.</td>
</tr>
<tr>
<td>Chelae odour source contact</td>
<td>Making contact with, seizing, or mounting the odour source with the chelae.</td>
</tr>
<tr>
<td>Meral spread</td>
<td>Chelae raised from the substratum and held apart.</td>
</tr>
</tbody>
</table>
Table 3. Proportion of total time spent performing behavioural responses to conspecific odour sources.

<table>
<thead>
<tr>
<th>Chela Treatment/Odour Type (N =10)</th>
<th>Chela(e) Waving</th>
<th>Chelae open/close</th>
<th>Chelae open</th>
<th>Chelae odour source contact</th>
<th>Meral spread</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact/ female</td>
<td>0.61&lt;sup&gt;1a&lt;/sup&gt;</td>
<td>0.12&lt;sup&gt;2a&lt;/sup&gt;</td>
<td>0.16&lt;sup&gt;2a&lt;/sup&gt;</td>
<td>0.10&lt;sup&gt;2a&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;2a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blocked/ female</td>
<td>0.23&lt;sup&gt;1b&lt;/sup&gt;</td>
<td>0.74&lt;sup&gt;2b&lt;/sup&gt;</td>
<td>--</td>
<td>0.02&lt;sup&gt;12a&lt;/sup&gt;</td>
<td>--</td>
</tr>
<tr>
<td>Intact/ male</td>
<td>--</td>
<td>0.94&lt;sup&gt;1b&lt;/sup&gt;</td>
<td>--</td>
<td>0.06&lt;sup&gt;1a&lt;/sup&gt;</td>
<td>--</td>
</tr>
<tr>
<td>Blocked/ male</td>
<td>--</td>
<td>0.10&lt;sup&gt;1a&lt;/sup&gt;</td>
<td>0.33&lt;sup&gt;1a&lt;/sup&gt;</td>
<td>0.42&lt;sup&gt;1b&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;1a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intact/ water</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Blocked/ water</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

-- = behaviour not observed

Different numbers (1,2) denote significant differences (P < 0.05) across rows
Different letters (a,b) denote significant differences (P < 0.05) across columns