A Test of Female Mate Preference in Relation to Male Dominance in the Crayfish *Orconectes quinebaugensis*

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Abstract

Male crayfish *Orconectes quinebaugensis* compete for dominance, therefore, we hypothesized that male dominance correlates with female mate choice, resulting in dominant males being chosen for more reproductive encounters. It was found that the dominance score earned by each male was significantly correlated to its claw size and that there was no significant correlation between the time females spent in the male-containing arm of a Y-maze and their time spent in a control arm. Analysis of our data indicates no relationship between a male's dominance and a female's mate preference.

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1 Introduction

In this project, the crayfish species *Orconectes quinebaugensis* sp. nov. (Mathews & Warren, unpublished data) was used as a model organism to study the evolution of social behavior. In crayfish, females are sexually selective because they bear most of the costs of reproduction, while males compete with one another for dominance status. However, the role of male dominance in female sexual selection has not been exclusively studied. The goal of this project was to determine if a correlation exists between male dominance status and female mate selection. In aquatic animals such as crayfish, chemosensory communication occurs through the release of chemicals called pheromones into the water. There are several kinds of pheromones including those associated with social status, reproductive state and dominance status. We hypothesized that virgin female crayfish prefer highly dominant males over subordinate males and would thus show greater attraction to the pheromones released by more dominant males. The subsequent sections provide background research on the following topics regarding crayfish; sexual selection, dominance and reproductive anatomy.

1.1 Theoretical Background Relating to Sexual Selection

Darwin's idea of natural selection emphasizes the preservation and modification of advantageous traits through evolution and genetic inheritance (Darwin, 1859). While natural selection depends upon the overall success of a species, sexual selection pertains specifically to the success of individual members over one another in the same gender (Darwin, 1872). This form of natural selection is not a direct competition of all individuals in a population for survival but rather a competition between members of the same sex in respect to reproduction (Darwin, 1872). Variation between individuals, therefore, is the driving force of selection and reproductive success (Darwin, 1872). Sexual selection encompasses several mechanisms including parental investment, mate choice, inter-sexual selection and intra-sexual selection.

Parental investment is defined as the cost endured by a parent to optimize the chances of offspring survival (Trivers, 1971). Females tend to have higher parental investment because their sex cells are less abundant and are typically larger than male sex cells (Trivers, 1971). Females have a finite number of sex cells which limits the amount of offspring that they can produce in their lifetime. Additionally, the physical and metabolic investment by a female to produce sex cells is much greater than the investment by a male. Due to lower energy requirements in males during mating, male reproductive success depends on the number of fertilizations in a lifetime rather than the number of sex cells produced (Trivers, 1971). As a result, females incur a higher energy cost in mating and have a tendency to be more cautious with each reproduction. Females

in a species tend to be choosier than the male for mating choice and therefore female choice directly affects sexual selection in males.

Poor mating choices may negatively affect a mating female. In a situation where there is unequal parental investment, desertion by the less investing parent is highly tempting (Trivers, 1971). As a result, females need to be cautious of males who are likely to abandon them after fertilization. Additionally, as seen by unequal parental investment, females have a limited number of sex cells and therefore decreased possibility of producing offspring with each mating. Errors in mate choice therefore could also lead to loss of time and energy by the female (Candolin, 1999). By making better mating choices, a female could increase her chances of producing more viable offspring.

Although dominance does not directly determine mating choice through forced copulations, it is thought to greatly influence female choice (Moore et al., 2001). Female choice is important due to direct and indirect benefits that a female gets from males. Direct benefits include food, parental care, and shelter while indirect benefits include beneficial genes inherited by offspring. Females tend to choose dominant males over subordinate males for direct benefits since they control larger amounts and higher quality of resources, including food and shelter (Qvarnström & Forsgren, 1998). Dominant males are more likely to protect the females due to their better fighting abilities. By choosing a male with resources, the female may better provide for her offspring. Additionally, dominant males have been found to provide better parental care (Qvarnström & Forsgren, 1998). Moreover, dominance may depend upon the condition of the male at times of competition. Higher dominance, therefore, can indicate better indirect benefits due to overall superior male condition including health, by suggesting he will produce viable offspring (Qvarnström & Forsgren, 1998). More dominant males are often a better mating choice, which suggests that male competitions directly relate to female choice (Moore et al., 2001).

While females are limited with respect to reproduction by their physiology, males are limited by their ability to attract and mate with members of the opposite sex. Males, therefore, typically maximize their fitness by increasing the number of mating opportunities in a lifetime. These changes in males tend to be affected by different classes of sexual selection; intra-sexual and inter-sexual selection. In the presence of mate choice during intra-sexual selection, one sex competes directly with members of the same sex to gain mates from the opposite gender (Krebs & Davies, 1993). When females are the deciding sex for mate choice, males compete with each other demonstrating their fitness in terms of their ability to produce viable, fertile offspring (Smith & Smith, 2001). In addition to competing to impress females, males may fight to keep others males away from a specific location, such as where a female is present (Moore et al., 2001). Dominant males, therefore, would increase the number of female mates by winning competitions. Different competitions have emerged as methods for males to display their dominance. Ardent males are those that participate in dramatic obvious competitions with one another for mates. These competitions can include fighting or ritualized displays between the two males (Krebs & Davies, 1993). Darwin(1872) theorized that sexual

selection affects weapons, body size, and plumage patterns by choosing traits found in these dominant males, and therefore more dominant males would have characteristics desired by choosey females.

During inter-sexual selection, members of one sex are attracted to members of the opposite sex based on different traits (Krebs & Davies, 1993). By avoiding competitions, the overall energy cost for a male to reproduce is lowered. To reduce the energy cost of competition, males have developed physical attributes as indicators of their dominance. Physical structures used to attract females are known as ornaments and may also serve as armaments which are attributes used in competitions and/or as dominance status badges to other males (Berglund et al., 1996). These ornaments, therefore, most likely evolved as armaments and later served as indicators of fighting ability due to inheritance from dominant ancestors (Berglund et al., 1996). Darwin (1871) explained that these desired traits are inherited over several generations since the victor of a battle is allowed to breed and these physical attributes are contained in the winner's genes.

Although inter-sexual selection and intra-sexual selection affect physical structures separately, some exaggerated structures have evolved to serve dual purposes (Berglund et al., 1996). Some evolved structures are involved in reproduction, while those affected by sexual selection may serve as cues to attract females and/or as weapons in competitions (Berglund et al., 1996). These characteristics not directly related to the reproductive system are called secondary sexual characteristics (Smith & Smith, 2001). The "good gene theory" suggests that females will choose mates based on these secondary structures because they correlate to overall fitness (Kirkpatrick, 1996). These structures, therefore, are preferred by females and become exaggerated overtime to attract mates (Kirkpatrick, 1996). Additionally, the "sexy son theory" suggests that females will choose males with these preferred traits in order to pass these genes to their male offspring (Cameron et al., 2003). By giving their son's genes that develop traits preferred by females, they will increase their son's mating opportunities.

An example of this would be the use of chemical signals called sex pheromones. In general, pheromones are chemicals released by one individual and detected by the chemosensory organs of other individuals. Chemical information is used to locate both food and mates, avoid predators, navigate through the surroundings and engage in social interactions. Social communication through pheromones can provide information to the conspecifics on genetic relationships, mate choices, territoriality, and social hierarchies (Bergman & Moore, 2005). Specifically, males release sex pheromones to attract females.

1.2 Female Mate Preference Studies

Several studies have been done to investigate male dominance levels and access to resources such as food, shelter, and female choice. Herberholz et al. (2007) investigated resource access within dominance hierarchies. Their results showed a

significant correlation between higher dominance and larger amounts of food resources. This suggests that dominant male *Procambarus clarkii* crayfish do in fact have more resources. Females, therefore, would benefit from mating with dominant males because they would presumably gain access to better resources.

Fero et al. (2006) studied the effects of male dominance on resource accumulation. In the presence of other *O. rusticus* crayfish, larger males obtained shelter less frequently, most likely due to lack of motivation for shelter. In the absence of other crayfish, however, larger males obtained shelter significantly more often than subordinate males. Additionally, mating was unaffected by male dominance. Another study by Nakata and Goshima (2003) tested the effects of size on acquisition of a residence from another crayfish and retention of a prior residence. Unlike Fero et al., this study showed that size played a large role in the accumulation of resources. Therefore, there may be discrepancies between different species of crayfish.

Other studies have been conducted to investigate the ability of female crayfish to detect differences among male crayfish. Stebbing et al. (2003) confirmed the presence of sex pheromones in *Acifastacus leniusculus* crayfish. Mature female sex pheromones were released into water with a male and the males were observed for mating behavior. The males were seen to positively react to the sex pheromones. These results suggest crayfish are capable of detecting the presence of other crayfish and distinguishing crayfish gender. In addition, Zulandt Schneider (1999) studied *P. clarkii* for pheromone release as an indicator of dominance status. This study observed male-male interactions for recognition of male dominance and indicated that dominance can be communicated through the use of pheromones.

Female crayfish have the ability to distinguish between male mates based on size which has been shown to have varying effects on the reproductive process. Gherardi et al. (2006) revealed that larger *Austropotamobius italicus* male crayfish have an advantage during non-random mating. In this study, larger males were seen to obtain mates more often and for longer periods than smaller males. These results indicated a female preference for larger males. Galeotti et al. (2006) tested the differential maternal allocation hypothesis in the freshwater crayfish, *A. italicus*, by evaluating the effects of specific male traits on female primary reproductive effort. The results showed that females laid larger but fewer eggs for relatively small-sized, large-clawed males and smaller but more numerous eggs for relatively large-sized, small-clawed males. This evidence suggests that females can adjust their level of reproductive investment in response to male traits, thus facilitating females to exhibit mate preferences in terms of egg size (Galeotti et al., 2006). By producing larger and fewer eggs, the female is investing more and expecting higher viability in offspring. This has also been shown to be the case in other species including the mallard duck (Cunningham & Russell, 2000). Individual female mallards (*Anas platyrhynchos*) lay larger eggs after copulating with preferred males and smaller eggs after copulating with less preferred males. This results in females producing offspring of higher fitness when paired with preferred males. Kotiaho et al., (2003) have shown that dung beetles also exhibit variable reproductive investment. Experiments done with the horned dung beetle (*Onthophagus taurus*) directly

compared genetic sire effects with maternal effects. They found strong evidence that mothers provide more resources to offspring when mated with large-horned males. This suggests again that differential maternal effects may amplify genetic effects on offspring traits that are closely related to fitness.

## 1.3 Crayfish Functional Reproductive Anatomy

Analysis of crayfish sexual selection relies on a thorough understanding of the functional anatomy of adult crayfish. Functional reproductive anatomy provides information regarding not only the physiological differences between males and females but also their differences in offspring investment and the success of the species through genetic variation by adaptive selection. It also lends to explanations of differential allocation and dominance behaviors.

There are two morphological forms of male crayfish; form I (F1) is the reproductive form in which males are sexually active, whereas form II (F2) males are sexually inactive. Form I males can be distinguished from form II males by an alteration in their first set of pleopods, tiny swimmeret appendages located centrally at the base of the abdomen.   This modification in a F1 male's pleopods allows the appendages to function in sperm transfer. Form, however, does not necessarily identify the age of a crayfish because mature males molt from F1 into F2 in mid-June and then molt again back to FI in early August (Weagle & Ozburn, 1972). In females, the seminal receptacles of mature females have two pronounced tubercles on either side of the anterior groove, while the seminal receptacle of immature virgin females is flat as indicated in (1).

The mating season for crayfish is in the early autumn. During forced copulation male crayfish insert their spermatozoa into the female (Huxley, 1973). The female stores the sperm until the spring. When her eggs are ready to be fertilized, the sperm and eggs are

Figure 1: Female Crayfish Tubercles

(Stein, R., Murphy, M., & Magnuson, J. 1977)

released simultaneously and external fertilization occurs. The fertilized eggs then attach to the swimmerets on the underside of the female's abdomen where the newly hatched crayfish receive needed protection from surrounding predators. The juveniles cling to the setae with their chelae and obtain food from their yolk sac until they become more aggressive and start foraging (Tal et al., 1999). After molting three or four times the juveniles leave their mother (Hobbs & Jass, 1988). Females during this time are not

cannibalistic, but are much more aggressive in the defense of their offspring, which is extremely common in most species (Figler et al., 1995). During this time the male plays no role in the investment of offspring.

Female choice may occur as "differential maternal allocation" by which females finely adjust their parental investment according to the characteristics of their current mate, specifically its attractiveness, and the likelihood of finding a better mate in the future (Sheldon, 2000). Differential allocation could arise since attractive mates transmit genes that will increase the fitness of their offspring. There is a general consensus that females should choose high status individuals as mates as pointed out by Borgia (1979), who notes that male success in fights summarizes lifetime success in collecting food, resisting disease, and avoiding predators and injury (Berglund et al., 1996). In addition, if the trait is heritable, females selecting mates for their fighting ability will have progeny that on average reproduces more successfully (Alexander, 1975). It has also been shown experimentally that female crayfish adjust their primary reproductive effort according to multiple male traits such as female crayfish producing larger eggs but smaller clutches when paired with relatively small-sized, large-clawed males, and larger clutches of smaller eggs for relatively large-sized, small-clawed males (Galeotti, 2006).

This in turn will result in variability by mate choice, which also has been shown in several other species on the basis of dominance. For instance, Howard & Minchella (1990) suggested that co-evolutionary cycles between hosts and parasites are more important in male-male competition than in mate choice. It can also been seen in *Drosophila*, where success in male-male competition for territories showed a considerable genetic variation (Hoffmann, 1988), in female honeybee workers where dominance had a high heritability (Moritz & Hillesheim, 1985), in three-spined stickleback aggressiveness and dominance were both variable and heritable in wild populations (Bakker, 1986), and in the cockroach *Naupeta cinerea* social dominance showed moderate to high levels of genetic variance (Moore, 1990). In all of these species, dominance has played an important role not only in their personal survival but also that of future generations.

1.4 Crayfish Dominance Behaviors and Competitions

Dominance status in a population specifies an individual as being more dominant or less dominant depending upon the consistency with which they win agonistic bouts (Bergman & Moore, 2005; Zulandt Schneider et al., 1999; Zulandt Schneider et al., 2001). This means that a more dominant individual will win bouts more consistently than a less dominant individual. Agonistic competition results in a reduction in the overall strife for resources within a population by establishing a "peaceful if uneven" distribution of resources (Issa et al., 1999). This social structure is maintained through displays and chemical signals which allow for fewer highly aggressive instances, thereby diminishing the cost of such interactions.

Crayfish are a widely used model organism for agonistic interactions. Previous studies have shown that competition is frequent in crayfish and that both males and females engage in agonistic interactions for shelter (Figler et al., 1999). The natural studies on crayfish indicate that the intensity is much less and duration is much shorter for competitions in the wild than those observed in a laboratory setting (Karnovsky, 1989). This could be due to the size of the area in which the bout takes place and the distance available for the passive crayfish to move away from the more dominant crayfish (Hediger, 1950). Males tend to engage in agonistic behaviors more frequently and intensely in the presence of resources (Nakata & Goshima, 2003; Figler et al., 1995). In the lab however, some species of crayfish have been observed engaging in agonistic bouts in the absence of any tangible resource other than space (Karnovsky, 1989). Their engagement in agonistic bouts in the absence of extrinsic factors allows for the observation of agonistic bouts while controlling for those variables (Heberholz et al., 2007).While females tend not to fight as intensely for resources as males, they will fight for shelter and they can become especially brutal when they are in their maternal phase. Further, male-female agonistic interactions tend to be less intense than male-male or female-female agonistic interactions (Ameyaw-Akumfi & Hazlett, 1975; Hazlett, 1985).

Although agonistic bouts primarily concern resources, intrinsic factors are also thought to play important roles, specifically for crayfish individuals who have participated in multiple encounters (Moore et al., 2002). One example of an intrinsic factor is the winner effect, which postulates that the more bouts an individual wins, the more likely they will be to win future bouts. The converse has also been observed; known as the loser effect, it is thought to result from a change in the losing crayfish's physiological response to chemosensory information released by a dominant crayfish into the water through urine (Zulandt Schneider et al., 1999). While the winner/loser effect could be misrepresented by random encounters between males of varying dominance or health status, it has been observed that when juvenile crayfish of the species *Procambarus clarkii* lose bouts, physiological changes in their neural circuit responses make them more prone to subordinate behaviors, such as tail flips which propel the crayfish away from their aggressor (Herberholz et al., 2001).

Genetics have also been postulated as an underlying dynamic aiding in the determination of agonistic bouts (Moore et al., 2002). The chemosensory signals, also called pheromones, excreted in the urine may be linked to the animal's specific genetics (Zulandt Schneider et al., 1999; Bergman et al., 2003). The signals provide immediate information about certain status aspects of an individual. Breithaupt and Eger (2002) showed that urination occurs deliberately during fights and that it happens much more frequently in males who win the agonistic interaction. They also were able to observe that urination increased during agonistic interaction as aggression escalated. Losers of previous fights gave up more quickly in successive bouts. Breithaupt and Eger (2002) found that chemical signals in urine act as a threat, which cause a decrease in the aggressive tactics of an opponent. Therefore, increased losing patterns may be explained by Bergman and Moores' (2004) prediction that exposure to dominant signals may result

in the release of the receiver's own chemicals, thereby exhausting their supply and diminishing a fighting tactic.

During agonistic bouts crayfish exhibit a number of ritualistic, often escalating behaviors (Bruski & Dunham, 1987*).* Ritual behaviors include actions such as attacking, retreating, and meral spreads. When attacking, crayfish aggressively use their claws (personal observation). Typically, the target crayfish responds by raising their claws and engaging in grappling. Raised claws are referred to as a meral spread in which a crayfish splays its chelae as illustrated in (2). A male crayfish will often exhibit a meral spread to another male in order to illustrate his fighting ability. The release of chemical signals and visual cues are thought to have a huge impact on the proceedings of a fight. When chemical signaling is inhibited bouts become more intense and long, the same is true in the absence of visual cues (Breithaupt & Eger, 2002; Zulandt Schneider et al., 2001).

Figure 2: Meral spread displays during an agonistic encounter between two crayfish.

(Bowling Green State University, 2007)

1.5 Hypothesis

The crayfish species used in this study, *O. quinebaugensis*, makes an excellent model organism for the study of chemoreception and its effects on male-female interactions in marine invertebrates. Orconectid crayfish can use chemical signals as a way to attract possible mates and convey dominance status (Adams & Moore, 2003, Bergman & Moore, 2005). Female orconectid crayfish will respond to chemical signals from male conspecifics with different levels of interest, depending on the dominance level of the opposing crayfish (Zulandt Schneider et al., 1999). Species of crayfish have also been shown to establish dominance through agonistic bouts (Adamson & Edwards, 1999). With their ability to communicate dominance status, *O. quinebaugensis* are a good model organism for testing the hypothesis that female crayfish prefer more dominant males. We tested this hypothesis with a Y-maze apparatus for detecting responses to distance chemical communication. It was predicted that a female in a Y-maze would spend more time in an arm containing a dominant male (versus a control arm) than in an arm containing a subordinate male (versus a control arm).

2 Materials & Methods

2.1 Crayfish Collection

Crayfish were collected from the Quinebaug River in Sturbridge at the Westville and East Brimfield Dam sites during the months of September and October. All of the crayfish collected had both chelae of approximately the same size, all of their legs and the

majority of their antennae intact. The males collected were all Form I. According to a previous study, virgin female crayfish prefer male conditioned water and non-virgin females show no preference for male conditioned water (Martin & Watkins, 2007). Therefore, in order to conduct this project only virgin females were collected. Approximately fifty males and one hundred females were collected.

2.2 Observations

After being collected, measurements were taken on all of the crayfish. Females were measured for their carapace length. Males were measured for their carapace length and the length of the right claw propodus. Based on these measurements the males were divided into two size classes. The small male group consisted of males with a carapace length up to thirty-seven millimeters, while the large group contained males with a carapace length of thirty-eight millimeters or greater. An average was taken for each group. The males chosen for experimental use were selected if they had a carapace length within ten percent (plus or minus) of the average. From those that qualified, males were additionally selected to ten percent (plus or minus) of the average right claw length for that group.

In addition to conforming to size requirements, only males with all walking legs and chelae of the same size were considered for selection. In total, twenty-eight males were chosen for experimental use, twelve that classified as small males, and sixteen large males. There were eleven replacement males kept on reserve for substitution if a selected male died. The replacement males were held to the same physical specifications for the group they qualified to substitute for.

2.3 Crayfish Care

Figure 3: Water system used to house crayfish

Three re-circulating water systems were used to house the crayfish. Each system circulated carbon, ultra-violet, and biologically filtered freshwater to fifty-five four liter tanks kept at room temperature (3). Although crayfish are nocturnal animals (Moore & Bergman, 2005), they were maintained at a standard daylight/darkness cycle for practical experimental purposes. The cycle was consistent with seasonal changes in the environment. Each crayfish was kept in a separate tank that was numbered for identification and contained a clay flower pot for shelter. All the males were kept in system 1 and the females were in systems 2 and 3. The crayfish were fed in the morning three times a week on a diet that alternated between broccoli and shrimp pellets. A daily census was taken to assess the condition of the crayfish and to check for dead or molted crayfish.

A week prior to the start of the male tournaments, circulation in system 1 was shut off. This was done in order to isolate each crayfish to prevent any chemical communication from occurring through the water system between individuals during the experimental period. Likewise, the females in systems 2 and 3 were isolated a week prior to use in the Y-maze experiment. The water in each individual tank was manually changed to keep the water clean and remove any debris, waste, and uneaten food. Additionally, an air stone was connected to each tank to aerate the water. Water changes took place three times a week in the late afternoon on the same days the crayfish were fed. A siphon was used to remove any leftover food and crayfish waste, along with about one-third of the water in each tank. The tanks were then refilled with filtered tap water from a hose. When performing a water change, care was taken to shake off the siphon and hose in between tanks to try to minimize the transfer of any chemicals between crayfish.

2.4 Male Dominance Tournament

2.4.1 Setup

In order to establish a dominance score for each male, a male dominance tournament was set up among the experimental male population. It was predicted that when two male crayfish were placed together they would fight and that the larger male would be more likely to win than the smaller male. While the males' ID numbers were not randomly generated, each of the males was also randomly assigned a lot number that was used to pair the males. A random number generator (Random.org) was used to generate a random non-repetitive sequence of the lot numbers for the large and small groups. For each group the first two numbers were paired to fight, followed by the next two, etc. until all males were matched with a partner. There were four rounds of the dominance tournament, in which each male got to interact with another male. The rounds were held on a weekly basis for four weeks. A male never fought the same opponent more than once. If a male died a substitution male was used in its place solely to preserve the score of the remaining members of the trial group and the substitute was not scored.

Figure 4: Plastic tank with removable divider used to conduct male dominance tournament

For each round, the paired crayfish were placed in a tournament tank partitioned into two sections using a plastic divider (4) to separate the opponents. One crayfish was marked with a "X' on its back using a silver marker in order to distinguish the two crayfish. To initiate a round, the divider was removed from the tank and the crayfish were videotaped for ten minutes. After the ten minute period was over, the crayfish were

transferred back to their tanks and the water in the tournament tank was changed to remove the traces of any pheromones released by the crayfish during the previous round. Latex gloves were worn and hands were washed after touching a crayfish to prevent the transfer of any human or crayfish chemicals to the crayfish or water in the tournament tanks.

2.4.2 Scoring

After each round the results of the fights were scored. Three group members individually scored the videotapes of the tournament. The scorers had no knowledge of the ID numbers of the crayfish in each round to prevent bias. While watching the tapes the scorers looked for three behaviors: approach, grapple, and retreat. The crayfish that retreated the most during the ten minute round was deemed the loser. The winner of each round received one point and the loser received a score of zero. If no winner could be discerned, the round was considered a tie and each crayfish received half a point. The three sets of scores were compared for each round. Results in which all three scorers agreed were included. Additionally, those which held a two-thirds agreement were also included if the third person's assessment of the fight was a half a point away from the other two peoples' decision. For each male that participated in all four rounds, a final dominance score was calculated by adding the points received from each round. The cumulative outcome of the four agonistic interactions each male participated in was used to rank each of the males on a dominance scale of zero to four, with zero being all losses and four being all wins.

2.5 Y-maze Experiment

The Y-maze apparatus was chosen for this crayfish behavior study for numerous reasons. While the apparatus is simple in design, it can show a lot about animal behavior by looking at how a specimen explores and behaves in each arm of the maze. Y-mazes are commonly used in crayfish studies to analyze the response of different individuals to chemoreception. By looking at the amount of time spent in each arm of the Y-maze, it is possible to see whether a female crayfish is attracted to the chemical signals coming from an arm containing a conspecific (Martin & Watkins, 2007). Other studies of crayfish using a Y-maze have also shown that females are more attracted to the chemical signals of dominant males (Zulandt Schneider et al., 1999).

2.5.1 Setup

In order to test the hypothesis that females prefer males of higher dominance status, a Y- maze apparatus was used (5). Two Y-maze apparatuses were utilized that were available from a previous study conducted by Martin and Watkins (2007). The apparatuses were constructed of black PVC piping cut in half longitudinally, with a diameter of 9.53cm, held together by ABS cement. The base arm measured of the Y-maze measured 13.65cm in length and each experimental arm was 12.4cm long. The

apparatus was covered by a piece of clear plexiglass to prevent the crayfish from escaping. Each end of the three arms had a crayfish holding area created by chicken wire. The experimental arms, in which a male was placed, also had a piece of plastic PVC with holes in it to prevent physical and visual interaction between the crayfish. The chicken wire used for the holding area in the base arm was removable so that the female crayfish placed in the arm could be released into the maze at the beginning of each trial.

Two Y-mazes were utilized, each connected to a water source that was continuously filled with carbon filtered water from the water hose to keep the water flow constant. An electric pump was used to pump the water from the large bucket to a smaller bucket located above it. A standpipe was located in the small bucket which circulated water back to the large bucket. The water passed through rubber tubing connected to the arms of the Y-maze. The right and left arms of the Y-maze each received an equal and continuous flow of water which ran down the base arm and flowed out into a drainage basin (5).

Twenty-eight males and fifty-six females were used for the Y-maze experiment. In order for a male to qualify for use, it had to have participated in all four rounds of the dominance tournament and received a dominance score. In total, fifty-six Y-maze trials were planned in order to use each female once. The males participated in two trials each. The females were paired with a male using a random sequence generator (Random.org) and the trials were carried out in order of male ID number. Fourteen trials were held twice a week for two consecutive weeks. The males participated in trails that were spaced a week apart in order to allow for a seven day rest interval between the first and second trial. Due to male death and disappearance between the first and second round there were only 54 completed trials.

Figure 5: Schematic and photograph of Y-maze setup

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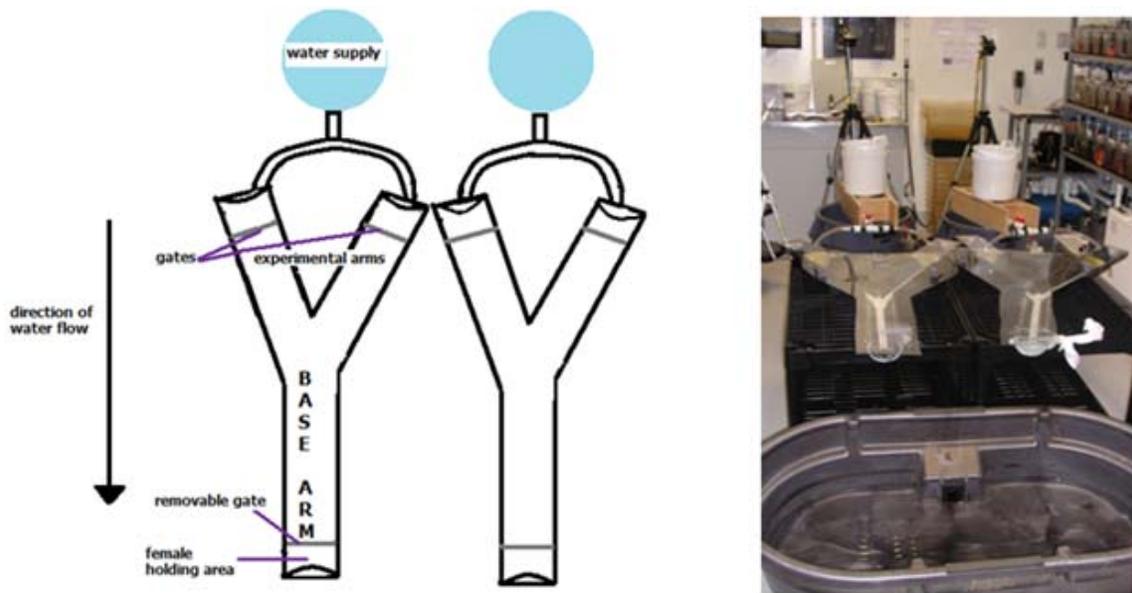
The Y-mazes were pre-tested with food dye to ensure that the water flow from each experimental arm mixed equally within the base arm, and did not flow back into the adjacent experimental arm. For each trial the male and female were added simultaneously. The female was placed in the holding area at the bottom of the base arm and the male was placed in either the right or left experimental arm. The side for the male was chosen using an electronic coin flip (Random.org) to eliminate female side bias. The crayfish were allowed a ten minute acclimation period to adjust to the environment. After this time, the holding gate was removed and the female was allowed to explore the Y-maze for ten minutes while being videotaped. After the trial was over the crayfish were returned to their tanks and the Y-maze system was allowed to run for five minutes to flush out any remaining pheromones and ensure a clean maze for the next trial.

2.5.2 Scoring

After each round the trials were evaluated by one reviewer. The two experimental arms were deemed right and left from the perspective of the camera. Timestamps were recorded for when the female exited or entered any of the arms. A female was considered to have exited or entered an arm when the tip of her rostrum crossed the base of that arm. The area between the three arms was considered the neutral triangle and was the site of preliminary mixing. Time spent in this area was not directly recorded. Timestamps were used to determine the amount of time spent in each arm. The difference between the time spent in the male treated arm and the control water arm was analyzed with the dominance score of the male. If the female failed to leave the base arm within the 10 minute trial period, the trial was not used. Any trial in which either subject (the male or female) died within 10 days of the trial was excluded.

2.6 Data Analysis

2.6.1 Male Tournament Data

All of the data analysis was performed in Microsoft Excel (2003, 2007) and SPSS (version 7). In order to determine if there was a correlation between male carapace length and claw length, a scatterplot was created to visually examine the data. A Pearson's Correlation test was then used to statically address the prediction that the claw and carapace sizes of male crayfish are directly related. The statistical null hypothesis of a Pearson's Correlation states that there is no linear relationship between the two variables. This test was appropriate for the data because the samples were divided into two groups based on size, which disrupted the normal distribution of the overall population. The Pearson's correlation is the standardized covariance; the ratio of the covariance between variables and their standard deviations.   The correlation coefficient is bound by +/- 1, where 0 indicates no relationship, positive numbers have a positive relationship and negative numbers have a negative relationship. When a variable is compared to itself there is a correlation of 1, because the covariance and standard deviation are the same.

Significant data allows you to reject the statistical null hypothesis that the variables are independent. Therefore, significant values indicate that the variables being tested, a male's carapace length and claw size, are interrelated. An alternative would have been to use a Spearman's correlation, however, this test was deemed less appropriate because it utilizes ranked data and the data in our analysis were continuous.

Next, the relationship between male size and dominance was examined. This was accomplished through the use of scatterplots, Chi Squared tests and a Pearson's Correlation.

2.6.2 Female Y-Maze Data

To analyze the hypothesis that females prefer more dominant males, an Analysis of Covariance (ANCOVA) test was used to determine if female size and male dominance independently or jointly affected the time a female spent in the male arm of the Y-maze versus the control arm. The independent variables of this test were female size and male overall dominance score. Male size was not considered as an independent variable since it was statistically found that male dominance was related to male claw size. Additionally, males were not visible to females during the Y-maze; therefore, their size should not have affected the females' choices. Male dominance and female size were analyzed one at a time, and then jointly to determine their effect on the dependent variable. The dependent variable was the difference in time spent in the arms of the Y-maze (time spent in the male arm minus the time spent in the control arm).

3 Results

3.1 Male Tournament Data

Figure 6, which contains size information on the 26 males used in the study, illustrates that there was a strong positive correlation between male claw and carapace size. A Pearson's correlation was used to statistically analyze the relationship between male claw and carapace size. The correlation test showed that when the carapace length and claw size were compared the correlation coefficient was 0.868 ($p<0.01$), therefore the null hypothesis was rejected. A positive correlation exists between carapace length and claw size.
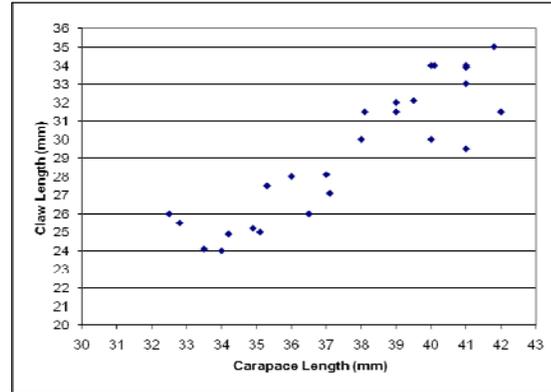

Figure 6: Comparison of male claw length to carapace length (Pearson's Correlation 0.868, $p<0.01$).

Although the males had different ratios of claw to carapace length, from 0.71 to 0.9 (Figure A in the Appendix), due to the direct relationship between male carapace and claw size, it was inferred that either variable could be used in analysis to represent the overall size of each male. Therefore, in subsequent analysis tests only claw size was used.

Figure 7 shows that when there is a large difference in claw size, the larger male is most likely to be the winner. There were only three instances where the male with smaller claws won an agonistic bout, and those instances all fell within the 0 and 1.5 mm difference range. In this analysis, all bouts from the 4 rounds of bouts which exhibit a clear winner and loser were used. Bouts which ended in a draw are not represented.
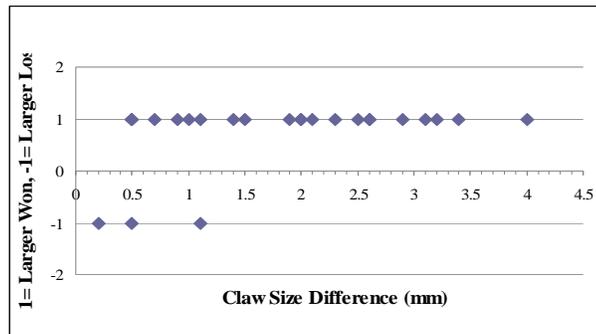

Figure 7: Relationship between claw size difference and bout outcomes for small paired males where there was a clear winner and loser, data from all 4 rounds.

A Chi-squared analysis was performed on the small male data to determine the likelihood of a crayfish with the bigger claw winning an interaction with another crayfish. The results showed that the observed instances where a crayfish with a larger claw won an interaction occurred more frequently ($X^2 = 14.44$, p= .0001, p< 0.005) than would happen by random chance if the null hypothesis were true. Only bouts from all four rounds with

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a clear winner and loser were used in this analysis. The results for the chi-squared analyses were checked using internet chi-squared calculator at www.psych.ku.edu/preacher/chisq/chisq.htm.
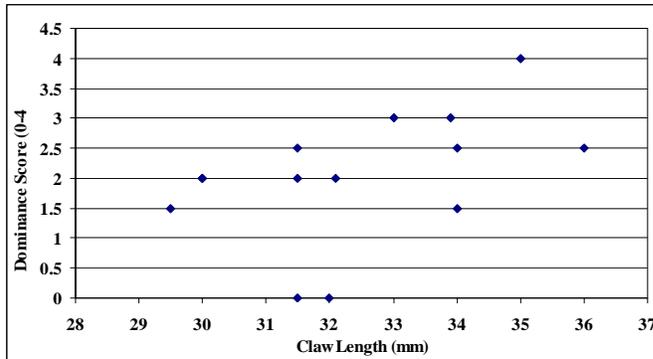

Figure 8: Claw length versus dominance score for the large male group (Pearson's correlation 0.459, p>0.01).

The graph in Figure 8 of claw length versus dominance score for the large males is not conclusive concerning the relationship between the two variables. A Pearson's correlation was used to analyze the assumption that males with larger claws are more dominant than males with smaller claws. The correlation coefficient for this test was 0.459, which indicates a slightly positive relationship, however, due to the significance level being greater than 0.01, the null statistical hypothesis cannot be rejected. Therefore there is no significant relationship between male claw size and dominance for the large male group.
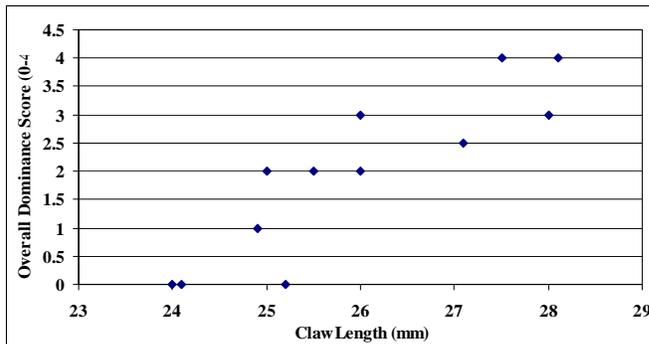
Figure 9: Claw length versus dominance score for the small male group (Pearson's correlation 0.884, p<0.01).

In Figure 9, the data for small males displays a positive relationship between claw length and dominance. The Pearson's correlation index for this data was 0.884, statistically significant at the 0.01 level which indicates a relationship between a small male's claw size and the likelihood of winning a bout. Thus, small males with larger claws have a greater chance of winning an agonistic bout.

3.2 Y-Maze Data

The following results are a test of the hypothesis that females prefer dominant males over subordinate males, using an ANCOVA. An ANCOVA analyzes the interactions between independent and dependent variables and it also allows the use of covariates. In Figure 10, negative numbers indicate that the females spent more time in the control arm than the male arm. The greatest positive time difference was observed for males with a dominance ranking of 2.5. Additionally, the number above each bar indicates the number of males or sample size of that particular dominance score (the number of males contributing to that time difference mean).
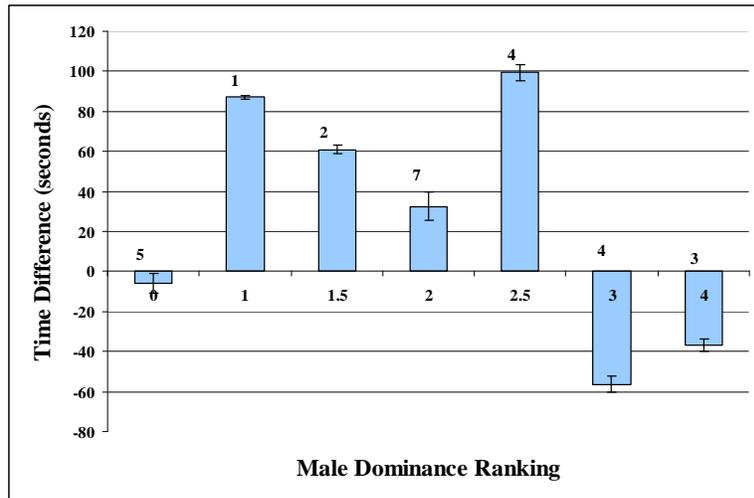
Figure 10: Time difference data (amount of time that a female spent in the arm containing a male minus the control arm) for each dominance score group. Numbers over bars indicate sample size (the number of females tested against males of that dominance score).

Figure 11 represents two rounds (combined) of Y-maze testing for large and small male data. The graph shows the qualitative relationship between male dominance and time difference. A negative time difference indicates that the female spent more time in the arm which did not contain a male. No direct relationship between the variables is apparent.
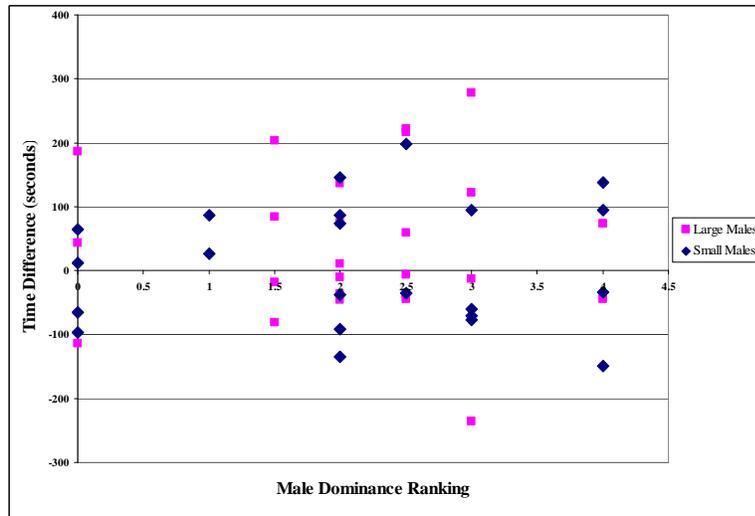
Figures 11: Male dominance ranking for males from two rounds of Y-maze testing versus the time difference for female location coded by male group (large and small).

Two ANCOVAs were performed. The first ANCOVA shows that the independent variables, male dominance and female size, do not interact to produce an effect on the time a female spends in the male arm of the Y-maze. The ANCOVA in Table 1 indicates that there are no interactions between the independent variables of female size and male dominance on the time difference (significance = 0.087, F > 1). Table 1 also reveals that independently neither female size nor male dominance affected the amount of time females spent in the male containing arm since all the p values for significance were greater than 0.01.

Source	F	Sig.
Intercept	2.095	0.169
Male Dominance	2.423	0.092
Female Size	2.962	0.109
Male Dominance x Female Size	2.474	0.087

Table 1: ANCOVA of the effects of male dominance, female size and an interaction term between male dominance and female size on the difference in time females spent in the treatment arm and the control arm.

Therefore, a second ANCOVA was then performed which did not include analysis of interaction between female size and male dominance.

Source		F	Sig.
Intercept	Hypothesis	.004	.953
Female Size	Hypothesis	.032	.860
Male Dominance	Hypothesis	.800	.582

Table 2: ANCOVA excluding an interaction term for male dominance X female size (no significance all p-values > 0.01)

For the second ANCOVA (Table 2) the p values for significance were all greater than 0.01. As a result, the null statistical hypothesis could not be rejected. It was concluded that dominance does not affect the amount of time that a female spends in the arm containing a male versus the time she spends in the control arm.

4 Discussion

Female selection may not be based on male dominance status. In this study, we tested the hypothesis that female crayfish will prefer more dominant male crayfish. Measurements of male crayfish in this study revealed that there is a positive relationship between claw and carapace size (Figure 6), which allowed for the use of only claw length in our analysis of size and dominance. Crayfish are known to engage in agonistic encounters and other studies have shown that size may play an important role in the outcome of agonistic interactions between crayfish (Figler et al., 1999). It was shown that larger male crayfish are more likely to win agonistic bouts and therefore we presumed larger crayfish would receive higher dominance scores in our study. The series of agonistic bouts performed with our male crayfish showed that as the size difference between the two competing males became larger, smaller males won fewer bouts (Figure 7). The statistical analysis showed that the relationship between size and dominance was not significant for large males, however, it was significant for small males (Figure 8 & 9). It is assumed that this occurred because the large males were more evenly size-matched during their bouts. Although size was statistically correlated with a male's dominance score, size information may not have been conferred to the female through chemical signals. Size seems to be less important in female mate selection because it can vary through time and it is not necessarily a good indicator of genetic quality. Females likely use indicators other than size for determining the genetic quality of mates. It should be noted that in this study, the preference of females for male size was not tested, however, we did indicate a correlation between male size and dominance. The results of the agonistic interactions allowed us to establish dominance scores for each male in the study.

Y-maze testing showed no significant relationship between male dominance and female preference in terms of the time spent by a female in the male containing arm of the Y-maze (Table 1 & Table 2). This indicates that females crayfish may not select mates based on their dominance status which supports Qvarnström & Forsgren (1998) who reviewed works in which females of some species actually prefer males of lower dominance due to their lower levels of aggression. Our results did not support the hypothesis that females would spend more time in Y-maze arms with chemical signals from highly dominant males. It was then concluded that dominance did not have a significant effect on female choice. Female *O. quinebaugensis* may prefer males based on factors that were not tested for in this study, including male health and the visibility of the male to the female.

In this study, we were unable to control for all of the environmental factors which could have lead to biases and flaws. Environmental factors were necessary to control for because our specimens were taken from the wild and were not necessarily raised under uniform conditions and circumstances. Although we attempted to control for environmental factors by creating specific conditions for the study, there were many factors which were out of our control. For example, any previous dominance hierarchies which had been established by the crayfish in the wild were ignored, and each male crayfish started with an assumed dominance ranking of zero. Another obstacle was that it

was impossible to create equal living conditions for all the crayfish in the project room. Some crayfish did not get as much lighting in their tanks as others due to their positioning on the shelves (personal observation). In addition, the health of each crayfish was unknown throughout the experiments. Some of the crayfish caught may have been sick, while others might have become ill later on during the experiments. These factors could have had an effect on the behavior of male and female crayfish during both rounds of testing. One particular example that highlights the inability to determine crayfish health was male 37. During feedings and water changes it was often observed that male 37 looked lethargic and did not finish his food. During the agonistic bouts, however, male 37 was extremely aggressive and won every fight. On the other hand, actual illness during the male bouts may have decreased the likelihood of a male winning the bout and increasing his dominance score. Additionally, if a male crayfish was sick during the Y-maze testing, the illness may have made his chemical signals unappealing to the female, despite having a high dominance status.

If the dominance scores are flawed, then the Y-maze results will be correspondingly flawed since the dominance of each male crayfish is not properly portrayed in the data analysis. A way to improve on the male dominance analysis would be to raise male crayfish in complete isolation from each other in a laboratory setting. Although the results from lab-raised crayfish may differ from wild crayfish, raising the test specimens in the lab would allow for environmental factors to be more rigorously controlled than when using a wild caught sample. This practice would also allow for a more complete understanding of the history of each individual used in the study. Age, size, feeding behaviors etc. could be closely monitored through the life of the individuals.

Bergman and Moore (2004) showed that exposure to chemical signals greatly influences the outcomes of agonistic interactions in the crayfish *O. rusticus*. By isolating our male crayfish we had hoped to control for the effects of signal exposure. This was critically important to our study because, as Bergman and Moore showed, maintaining the animals on a flow-through water system could have caused our male crayfish to exhaust their chemical signal supply prior to both male-male interaction and the Y-maze. As pointed out by Breithaupt and Eger (2002), chemical signaling plays an important role in the agonistic interactions of crayfish. Using Fluorescein to make urine visible in the water, they showed that crayfish excrete urine (chemical signals) specifically during battles, and that they direct the flow of those signals differently during battle than when not in battle. Further, the winners of agonistic interactions are typically the crayfish who release signals and losers were found to concede more quickly in future battles. In our study, the history of each specimen was unknown, therefore the influence of chemical signals could have already had effects on our specimens. Herberholz (2001) showed that successive losses caused physiological changes in juvenile crayfish making them wired for submissive behaviors in response to dominant chemical signals. Although none of our crayfish failed to engage in interaction, these previous studies suggest a great deal of bias and control problems which may exist in our data as a result of collecting the specimens from the wild.

Barki et al. (2003) showed that male-like aggressive behavior and secondary sexual characteristics are connected to the androgenic gland. Implantation of an androgenic gland into an immature female crayfish resulted in male-like behaviors when interacting with an intact crayfish female. This paper established a chemical link to gender specific behaviors in crayfish. Chemical signaling could, therefore, also have played an important role in our Y-maze experiment. Although naïve female crayfish have previously been shown to spend more time in the arm of a Y-maze containing a dominant male crayfish (Zulandt Schneider et al., 1999) it is possible that males and females react differently to the same chemical signals. Breithaupt and Eger (2002) showed that the crayfish *A. leptodactylus* typically only urinate one or two times per hour. In terms of our Y-maze this could mean that the twenty minutes (ten acclimation and ten exploration) spent by a female in a Y-maze may not have been sufficient time for the male to release chemical signals.

The work of both Bergman and Moore (2004) and Breithaupt and Eger (2002) highlight the fact that both chemical signals and visualization of their opponents play a role in the agonistic interactions of crayfish. Our Y-maze experimental results, which may have been hindered by a lack of chemical signals flowing through the maze, may have been further marred by our controls. We specifically wanted to address female selection in terms of chemically signaled dominance status, therefore, we blocked the male crayfish from view in the Y-maze to isolate our variable of choice, chemical signaling. We assumed that the male would release chemical signals during a twenty minute period and that the flow-through water setup would not wash the signals through the maze too quickly for the female to react to them. If our assumptions were wrong and females select mates based on either visual cues or a combination of cues, this Y-maze setup may have been inappropriate for addressing female mate preference. For example, male crayfish may only urinate if they can see the female or a female may only be attracted to male signals if she can see the male. Concerning dominance, female crayfish may rely on secondary sexual characteristics of males rather than chemical signals. For instance, we found a correlation between male claw size and dominance, perhaps females are attracted to males with visually large claws but not necessarily dominant chemical signals.

Surprisingly few studies have been done on the importance of visual signals in mate recognition in invertebrates other than insect species. However, researchers Detto et al. (2006) found the first empirical evidence of the social importance of color markings in fiddler crabs (*Uca mjoebergi*). The researchers noted that certain species have a certain waving motion done by the males, which was believed to be involved in attracting a mate. To test for this, the researchers painted over the claw of one species of male to make it look like that of another. The results of this experiment showed that female *U. mjoebergi* used the coloration of the male's claws independently of their waving display to identify mates. Again, this data seems to indicate that visualization has an extremely important role in mate selection more so then other signaling patterns. Further, female butterflies have been found to rely heavily on visual cues when chemical cues are

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blocked meaning that both visual and chemical cues are equally important when selecting a mate (Costanzo & Montiero, 2007).

Male dominance ranking had no significant effect on the time a female spent in the male arm of the Y-maze. Although there may be no correlation between male dominance and female preference, there could have also been many faults in the experimental design that affected female preference. Defects in the experimental design may include set-up flaws, sidedness in crayfish, and the female not leaving the base arm (in this case the data were not included in analysis).

There were several aspects of the Y-maze set-up which could have resulted in insignificant or flawed data. The flow rate of each individual tube in the Y-maze could have affected the signals received by the female. The only test performed to measure water flow was a dye test. Accurate determinations of flow rates were not identified prior to experimentation. When a crayfish was placed in the apparatus it may have chosen a path based on water resistance as opposed to chemical signals. Further, the centrally located base arm may have adversely affected the female time data. The base arm received water flow from both arms, which may have confused the female when she was attempting to locate the origin of the chemical signals. If the flow of chemical signals from the male was constant, the use of a base arm would not have been problematic because the female would have been able to trace the chemicals to the source. The release of chemical signals from the male is assumed to be periodic, therefore any signals released may have been flushed out of the Y-maze with the water. Additional problems could pertain to either the concentration of signals or normal male-female interaction behaviors. For example, a female may only approach a male up to a certain distance during their mating rituals.

The structural design of the Y-maze may have posed further problems. Female crayfish in the Y-maze exhibited right side-bias (Warren, A., personal communication), meaning they preferred to turn into the right arm of the maze versus the left regardless of the presence or absence of a male in that arm. We attempted to control for side-bias by randomly assigning the males to either the left or right arm. Therefore, side bias should not have had an effect. Another problem occurred when a female remained in the base arm throughout the majority of the trial. Females who did not leave the base arm were subjected to a retrial. One possible solution to this problem would be waiting to start timing until the female leaves the base arm. This solution, however, would not account for a female who remains in the base arm due to presence of male signals carried in by water flow as previously stated.

Based on the results of this study, female *O. quinebaugensis* crayfish do not prefer males of higher dominance as conferred through chemical signals. Female crayfish are thought to invest more in reproduction, and are therefore likely to be choosier than the males. Our findings support previous studies in which it was found that females may prefer less dominant males. Dominance in crayfish may be a form of intra-sexual selection which reduces the pool of mates available to females and thereby ensures that a dominant male will be more likely to obtain mates regardless of female preference. In this study, it was also found that the claw and carapace sizes of male crayfish are

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related to their dominance in our small size bracket of males. Future studies could attempt to uncover any possible connections between male size and female preference, however, since male size varies throughout their lifespan, females likely base preference on other qualities which are better indicators of a mate's fitness. Further studies should also be done to explore other attributes that may be related to female preference such as chemical signal make-up, visual features; displays, physical traits or resources. A few suggestions for improvements to this particular study would be to use Fluorescein to visualize the chemical signals in the water and raise the crayfish in the lab so that we know as much of their history as possible. Additionally, it would be advisable to collect and store known samples of chemical signals from a specific organism for use in later behavior experiments. Our study sheds light on the fact that mate selection is a complex process, and raises new questions concerning the antiquated notion that the alpha male always gets the girl.

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