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# The role of pheromones in crayfish mating behavior: Responses of virgin and non-virgin females to conspecific chemical cues

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# The Role of Pheromones in Crayfish Mating Behavior: Responses of Virgin and Non-virgin Females to Conspecifics Chemical Cues

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By


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# Abstract

In this project, completed in conjunction with Worcester Polytechnic Institute's Biology department, we tested the hypothesis that virgin *Orconectes virilis* female crayfish would be attracted to water conditioned with male pheromones. We also hypothesized that the non-virgin females would not be attracted to the male conditioned water, as they had already mated that season. A y-maze was constructed and used to test the response of mated and unmated female crayfish to water conditioned by male or female conspecifics. The data showed that as predicted in our hypothesis only previously unmated females were attracted to water conditioned with male conspecifics.

## Acknowledgements

We would like to thank our advisor, Professor Lauren Mathews, for her continuous assistance and guidance throughout our project. We would also like to thank Professor Dan Gibson for the generous use for his lab space. Lastly we would like to thank Will Durgin for the use of his testing apparatus and experimental data.

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# 1. Introduction

Much research has been conducted into the evolution of social and mating behaviors of arthropods. This experiment explores the role of pheromones in the mating behavior of female *Orconectes virilis*. Specifically, we tested the hypothesis that not only can females detect male pheromones, but are also attracted to them depending on their mating status. Females who have not previously mated were predicted to be attracted to the male pheromones. We thought that this would happen, because females who had not previously mated should be actively seeking mates. This was tested by using a Y-maze to observe the response of virgin and non-virgin females to water conditioned with male or non-virgin female conspecifics.

## *1.1. Orconectes virilis*

While much research has been conducted on various crayfish species, very little is known about the species used in this experiment, *Orconectes virilis*. Many behavioral studies have been conducted in the laboratory setting, which does not always produce accurate accounts of the true behavior of the species. Therefore more and more studies have been done in natural settings using mainly observation as a way to learn more about these crayfish. Information about their habitat, size, color, molting, mating behavior, and mortality has all been collected through these studies.

A population of *Orconectes virilis* was found in a small town in Michigan, living in a stream and its connecting pond. The pond and stream had multiple microhabitats including in open water, under rock, under vegetation, and in burrow (Hazlett, Rittschof & Rubenstein, 1985). The crayfish were located and their size and habitat were recorded. Many of the crayfish were found inside burrows by themselves with rare instances of two crayfish sharing a burrow. The burrow systems were found in both loose soil and packed clay. The burrow systems were very extensive, often with multiple entrances and interconnections (Hazlett, Rittschof & Rubenstein, 1985). Both males and females were most likely to be found in burrows, but the females preferred the burrows more than the males. It was uncommon to find crayfish in the open water during the day, but

when collected at night, many more were found outside of their burrows (Hazlett, Rittschof & Rubenstein, 1985).

It is also common to find *Orconectes virilis* residing in crevices in rocks or in pits in river banks or under rocks (Bovbjerg, 1970). Fewer crayfish of this species are found in ponds compared to rivers and streams (Bovbjerg, 1970). Streams tend to be constantly aerated due to their movement, while ponds can become stagnant and oxygen levels fluctuate depending on the time of day and season. The lack of oxygen and variability in its levels probably accounts for why fewer *Orconectes virilis* are found in ponds (Bovbjerg, 1970).

*Orconectes virilis* play an important role in their habitat. They typically feed on a diet of detritus and macrophytes (Mitchell & Smock, 1991). They also almost completely comprise the diet of many bass species (Mitchell & Smock, 1991).

While most populations of *Orconectes virilis* have an average adult carapace length of 40-50 mm, the size can vary depending on the population (Hazlett & Rittschof, 1985). One population was found to have individuals with carapace lengths frequently over 55 mm, with a maximum length of 69 mm. These crayfish had the same survivorship rates as the smaller sized crayfish (Hazlett & Rittschof, 1985). Their larger size was probably only due to available food resources and lack of predation.

*Orconectes virilis*, like most crayfish, can vary in growth rate as well as size. The crayfish grow in size with each molt, usually only 1-3 mm, but they can grow up to 5-6 mm per molt (Hazlett & Rittschof, 1985). Their growth is most rapid in May and throughout the rest of the summer, with virtually no growth during the winter months (Mitchell & Smock, 1991). Females are less likely to molt in the early summer than the males. This is probably due to the fact that they are still carrying eggs and molt after depositing the eggs (Hazlett & Rittschof, 1985). Hazlett (1985) also found that as crayfish get larger, they are likely to molt less than smaller individuals.

It has been known for many years that male *Orconectes virilis* have multiple forms (Momot, 1967). However, it has recently been discovered that females of

this same species have multiple forms as well (Wetzel, 2002). Males have three different forms. The first is a juvenile form present before maturity. The second is a non breeding adult form (Form II). The last is Form I, which is characteristic of breeding adults (Momot, 1967). The females have only two forms. Form I, like the males, are sexually mature females and Form II are not (Wetzel 2002). The crayfish are usually mature after they molt in July (Momot, 1967).

Crayfish of the species *Orconectes virilis* mate in the fall, usually in October to November. The spawning occurs in the spring and lasts about 7-9 weeks (Muck, Rabeni, & Distefano, 2002). In the genus *Orconectes* spermatophores from males are stored in a seminal receptacle which can be sealed with a sperm plug (Walker, Porter, & Avise, 2002). The young are typically born in May and are independent in June (Mitchell & Smock, 1991). The recently hatched young stay in the shallow water while the mature females and males retreat to the deeper water (Momot, 1967).

After shedding their first brood, females are ten times more likely to die (Momot, 1967). The maximum lifespan for *Orconectes virilis* is two to three years with most only surviving about 18 months (Mitchell & Smock, 1991). Female crayfish were found to have a higher mortality rate than males (Momot, 1967).

Hazlett (1985) conducted a study of the species *Orconectes virilis* and the response of males and females to different conspecifics. It found that the females differed very little in their responses to all the different treatments. However, the males had very slight different responses to the various conspecifics. Males were able to distinguish between water inhabited by themselves and water inhabited by other males. They concluded that females may not be able to detect any differences in chemical signals, but males are able to (Hazlett & Rittschof, 1985).

Throughout the introduction, information about a variety of different species will be presented to use as a background for this experiment. Because there are not a lot of studies conducted on our species, *Orconectes virilis*, which are directly related to our hypothesis, we have researched studies involving a variety of other species. Many of these species vary at the level of species or genus; there

are also some that vary at the family level as well, particularly the lobster *Homarus americanus.* The divergence in genes between a species such as this lobster and northern crayfish species is actually no greater than that of *Orconectes* and *Parasticidae,* another family of crayfish (Fetzner, 2002). Even though there is divergence between these species, they still have many behavioral similarities that make them good reference species to study.

## *1.2. Chemical Communication*

Arthropods live in a world of chemical signals invisible to humans. These animals gather information from changes in chemical concentrations, flow direction, and chemical intermittency in the air or water. Using this information they are able to navigate and detect food, avoid predators, locate shelter, find mates and determine information about conspecifics.

This information is gathered in a number of different ways. It has been found that blue crabs use a method known as odor-gated rheotaxis (Weissburg, 1993). They travel along the outside of odor plumes and by using the direction of the flow as well as the intermittency of the chemical signal they are able to locate odor sources. Lobsters and crayfish do not use the same method. Rather than traveling along the edge of an odor plume these animals seem to position themselves in the middle of it. By doing this lobsters and crayfish use chemotaxis (Atema, 1996, Moore, 1991). Lobsters gather spatial and temporal information from the odor plume and this information leads them to the source. Crayfish appear to use only spatial information when orienting themselves and hunting for an odor source (Kraus-Epley, 2002).

Kraus-Epley (2002) conducted a study using *Orconectes rusticus* to determine the effects of antennae and antennules on the ability of the crayfish to accurately locate an odor source. They found that both bilateral and unilateral, partial and complete lesions impaired the animal's ability to locate the odor source. From this they concluded that the antennae and antennules detect information that is necessary for the animal to successfully search for food. Devine (1982) conducted a similar study using *Homarus americanus.* The effects of antennule

ablations as well as blocked leg receptors were examined. It was found that while the antennules played the main role in orienting the lobster, the leg receptors did have a role in the lobster's orientation.

By using information detected by their antennules and antennae, lobsters and crayfish are able to quickly and accurately locate food sources. Atema (1996) found that it took lobsters about thirty seconds to locate a food source two meters away. During the searching process, the animals walked slower and flicked their antennules. Considering the complexity of the environments the animals live in, finding a single food source in a laboratory setting should be fairly simple. In a natural environment there are many different odor sources, constantly changing flows, concentrations, and odor intermittencies. Wolf (2004) found that as the complexity of the odor sources increased (by adding a second food source), the speed at which the crayfish walked and their orientation accuracy actually increased.

Lobsters and crayfish have a complex social structure. These animals give off chemicals that indicate many different things about social status, including sex, molt status, and individual information about dominance (Hazlett, 1985, Karavanich, 1998, McLeese, 1970, Tierney, 1982).

Males in some crayfish species have been shown to be able to distinguish water conditioned with crayfish of different sexes. Male *O. virilis* respond much differently to water conditioned with male conspecifics than water conditioned with female conspecifics or self. Male crayfish were observed posturing aggressively when they detected male conditioned water (Hazlett, 1985). Some crayfish species are even able to distinguish between female conspecifics and females of different species. *Orconectes propinquus* and *Orconectes virilis* males, when tested against females of other species and conspecifics, were able to differentiate between the two females and were attracted to female conspecifics (Tierney, 1982). While *O. virilis* were able to distinguish females of different species solely by chemical signals, it was found that *O. propinquus* required other sensory information (visual, tactile, behavior). *Orconectes rusticus* and

*Orconectes obscurus* were found to have more trouble distinguishing between species and like *O. propinquus* used other cues besides chemical (Tierney, 1982).

Sex is not the only information that crayfish and lobsters can determine through chemical signals. Karavanich (1998) found that male lobsters who had previously encountered one another in an aggressive situation that resulted in a dominant and subordinate relationship recognized each other after 1-2 weeks of separation.

Just as it is important for the animals to be able to detect each other, it is also important that they have a defense mechanism to keep themselves safe from predators. Lobsters can limit their secretions to help camouflage into their surroundings, just as they can give off chemicals to indicate their presence (Atema, 1995). Lobsters store urine and use it at opportune times, such as during fights or when trying to attract a mate.

Not only do lobsters and crayfish respond to chemical signaling between members of their own species, but a study by Hazlett (1990) made some very interesting conclusions about interspecies signaling. He used three different crayfish species including *Orconectes virilis*, the species used in our own experiment. Each species was placed in water conditioned with different animals that the researcher had agitated, including other crayfish species, turtles, fish, and even leeches. While two of the species showed no reaction to the conditioned water, an alert response in *Orconectes virilis* was observed in reaction to water conditioned with all animals, except for the turtle. This could be because turtles have a different alarm signal.

It is clear that crayfish and lobsters can detect a significant amount of information from chemical changes in the water. The degree to which it is utilized by different species is not yet fully understood.

## *1.3. Polyandry in* Austropotamobius italicus

When a species practices polyandry, competition between sperm from all mates arises. This in turn produces many different adaptations that will enhance the sperm or counter the sperm from competing males. In the crayfish species

*Austropotamobius italicus*, the males have developed a characteristic to help ensure that their sperm fertilize the eggs of the females and not prior mates. The males feed on the spermaphores previously deposited by the male who had mated with the female prior to them (Galeotti et al, 2007).

As this is a unique practice of the crayfish species *Austropotamobius italicus*, Galeotti (2007) and his colleagues looked further into the mating habits of this species. They hypothesized that males should exhibit different behavior towards females who had previously mated and those that had not. They also predicted that because the males fed on the spermaphores already deposited, this would lead to last male prevalence paternity (Galeotti, 2007). Because of this, it was expected that males would increase the amount of sperm for females who had already mated to ensure that their sperm was the most prevalent (Galeotti, 2007).

Female crayfish were mated with two male crayfish sequentially to see the effects of polyandry on mating habits. The females did not show any difference in resistance to the first male that they mated with or the second. It was also found that males did not behave any differently as well nor did they expend more sperm when mating with females who had previously mated (Galeotti, 2007).

Almost all of the males who mated second removed the spermaphores of the other males before mating with the females. However, very rarely a male would simply add his sperm to the spermaphores already deposited. The males were successful in removing about 65-70% of the spermaphores deposited by the first male (Galeotti, 2007). They then added their own spermaphores to the spermatophoric plate, with their sperm now accounting for about 85% of the spermaphores. This means that the females who mated at least twice had an average of 30% more spermaphores (Galeotti, 2007).

Because the females who mated multiple times had more spermaphores attached to the spermatophoric plate they may have a higher fitness. If the number of eggs fertilized depends on the amount of spermaphores deposited, and the amount of spermaphores of on male is not enough to fertilize all of the eggs, then those females with spermaphores from multiple males have an advantage (Galeotti, 2007). This is one potential reason why it is beneficial for females to

mate with multiple males. However, the benefit for males is not as obvious. Males want to mate with as many females as possible because another male could come after him and remove his spermaphores. Ideally they would like to be the last male to mate with the female. However, since it is believed that they cannot tell when the female is about to lay the eggs, they cannot determine if they will be the last male to mate and have to take their chances.

Walker et al. (2002) looked at 15 females of the species *Orconectes placidus* and their clutches. During mating a sperm packet is deposited in a seminal receptacle in the female, which can be sealed with a sperm plug. Females have been observed mating with multiple males. In this experiment the parentage of 15 egg clutches was determined using microsatellite data. Out of the 15 clutches examined 6 of them were found to be from a single sire while the other 9 had multiple sires. The number of sires found for the multiple sire clutches ranged from 2 to 4. However, in all the but two of the clutches there was a dominant sire responsible for fertilizing as much as 85% of the eggs. In the two exceptions the clutch was split nearly equally between two males. The authors mention that it is possible that the clutches that were found to be sired by only one male, actually were by multiple males and the random sampling used in this experiment simply did not detect it. It was also concluded that the sperm plugs do not seem to prevent multiple inseminations.

## *1.4. Mating in other species*

While there has not been a lot of research done on *Orconectes virilis,* there has been a considerable amount of research done on many other species of crayfish and lobsters. This information allows comparisons to be made between conclusions made in previous studied and the new data found in this experiment.

### *1.4.1. Homarus americanus*

Premolt females of the species *Homarus americanus* choose a male and form their pair bond by repeatedly visiting his shelter. The females are the ones who are active in mate selection rather than the males. The mature females molt half an hour prior to mating; therefore female sex pheromones

are typically found in molt water. Atema and Cowan (1986) found that male lobsters responded strongly to female urine as well as female molt odor. However they did not respond strongly to male urine or male molt body odor nor did the females have strong responses to any of the body odors or urine. This shows that while the female lobsters actively seek mates, the males are able to determine when the females are ready to mate (Atema & Cowan, 1986).

A different study (Cowan, 1991) determined that the antennules of female lobsters play a critical role in reproductive behavior. Those females whose antennules were removed were less likely to cohabit with the males; they were also more likely to suffer injuries from molting under atypical conditions. The males whose antennules were removed allowed females to cohabit and mate as usual. There are a few possible explanations for why these differences happened. Perhaps females need to be able to detect the male odors before entering the male shelters for cohabitation. Or maybe the females need to identify the male conspecifics in order to produce their odors that the males detect to allow them to cohabit. The males who had their antennules removed were still able to mate normally, however they did injure the females more often than normal male lobsters. The possible reason for this is that the female odors may suppress male aggression towards the females (Cowan, 1991).

### *1.4.2. Pacifastacus leniusculus*

It is known that the crayfish species *Pacifastacus leniusculus* has seven distinct mating stages. They are orientation, contact, seizure, turning, mounting, spermatophore deposition, and dismounting. A study conducted by Stebbing (2003) was done in order to determine if females release a sex pheromone which the males respond to during the mating season. When water conditioned with mature females was added, the males spent more time handling the air-stone, specifically demonstrating the stages of seizing and mounting. The males showed no divergence from previous activity when the control water or the immature female water was added. They concluded that a

pheromone is released by a mature female during the mating season that stimulates male courtship behavior (Stebbing, 2003).

### *1.4.3. Orconectes rusticus*

In the species *Orconectes rusticus*, the male crayfish have two different morphotypes – reproductive and non reproductive. The reproductive males have long white reproductive stylets and more robust major chelae with more sensory hairs. In a recent study (Belanger & Moore 2006), it was determined whether or not the chelae were used for determining female sex pheromones in both reproductive and non reproductive males. The reproductive males whose chelae were intact responded the most to the female conditioned water, whereas the reproductive males whose chelae were blocked spent significantly less time handling the water source. Non reproductive males, regardless of chelae condition, did not respond to the female-conditioned water at all. Their results showed that in order for the male crayfish to identify the odor source, the peripheral chemosensory input from the chelae is required (Belanger & Moore, 2006).

## *1.5. Goals and Hypotheses*

In this experiment the role of pheromones in the mating behavior of female *Orconectes virilis* is explored. The females were tested using a Y-maze and given choices between control water and male conditioned water or non-virgin female conditioned water. Our hypothesis was that the female crayfish would be able to detect the male pheromones. Additionally, we hypothesized that the virgin females would be more attracted to the male conspecifics because they would be looking for mates.

# 2. Materials and Methods

## *2.1. Collection and Care of Crayfish*

The species *Orconectes virilis* was used throughout this experiment. The crayfish used in the experiments were all collected from local waterways, between the months of September and November, the majority of which were collected from the Quinebaug River in Sturbridge, Massachusetts. The crayfish were collected with handheld nets and by seines. The crayfish were divided into three types (males, virgin females, and non-virgin females) and housed in different tanks by type. The first sample of crayfish was collected in August, which was before the known mating season for *O. virilis*. During this time, no mating had been observed in the field or in the lab, therefore these crayfish were designated as virgin. Although these crayfish may have mated in previous seasons, they had yet to mate this season and for the purposes of this experiment, they were considered virgins. Females caught during the mating season for *O. virilis* and after mating was observed in the field or in the lab were assumed to be non-virgin. Males were collected both before and during the mating season and were not treated differently based on this fact.

Approximately 20 crayfish were housed in each tank. The tanks were filled with water filtered by a Marineland Penguin 350 BIO-Wheel Power Filter. Filter cartridges were changed monthly and water changes were performed weekly. Tanks were unheated and remained at room temperature. The room was kept on a natural day/night cycle. Clay flower pots were provided as shelter for the crayfish. They were fed Wardley shrimp pellets daily.

## *2.2. Apparatus*

In order to test our hypotheses, a Y-maze and apparatus was designed and built for this purpose. The maze needed to offer the crayfish a choice between two different water sources. The sources needed to remain uncontaminated while still allowing the crayfish to move freely between the two choices.

Figure 2.1 illustrates the basic setup of the apparatus. The two Y-mazes (Fig 2.1C and D) were connected to two reservoir containers (Fig 2.1A and B). The water from container A flows to the right arm of each maze (illustrated in red). The water from container B flows to the left arm of each maze (illustrated in blue). The water flows through the maze and out the end of the stem. There was a gate at the end of the stem, creating a holding area (Fig 2.1E) to allow the crayfish to acclimate to the water prior to the testing. The dashed lines in the center of each Y-maze designate the separations between the neutral zone of the stem, the decision zone of the middle, and the choice zone of each arm, all of which were used to analyze the data.

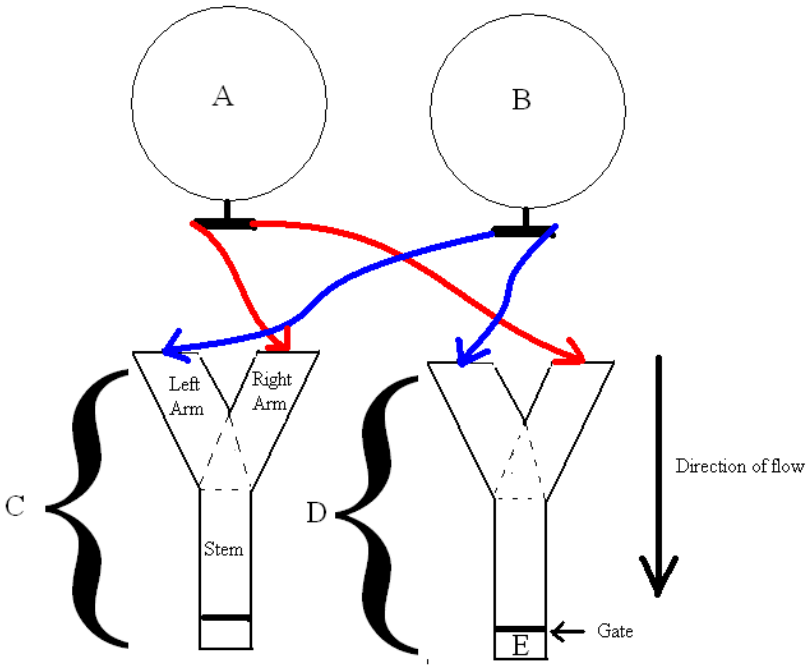


**Figure 2.1 Illustration of Y-maze** *The two Y-mazes (C and D) were connected to two reservoir containers (A and B). There was a gate at the end of the stem, creating a holding area (E).*

In this experiment, two identical Y-mazes were constructed to test our hypotheses. They were constructed of black PVC piping cut in half longitudinally, with a diameter of 9.53cm and connected with ABS cement. The stem of the Y-maze measured 13.65cm in length. Each arm was 12.4cm long and was attached to the stem at an angle. The Y-maze was covered in clear plastic so

the crayfish could not escape, but could still be seen from above. Chicken wire was installed at the end of each stem so that the crayfish would not fall out, but water could still exit. Additionally, chicken wire was used for a gate at the end of the stem of the Y-maze to contain the crayfish while it acclimated to the water flow prior to the start of each trial. This part of the apparatus can be seen in Figure 2.2.

**Figure 2.2 Picture of the Y-maze portion of the apparatus**

Two identical 56.8L plastic containers (Fig 2.3E) contained the water used for the experiments. The water was pumped with a HP electric pump from this container to a smaller 7.6 L container above it (Fig 2.3D). In the small container was a standpipe, through which the water could return to the larger bucket. This regulated the flow of water into the y-maze. From there, the water passed through a valve (Fig 2.3C) and into rubber tubing (Fig 2.3A and B) which was connected to the arms of the Y-maze. Figure 2.3 shows a diagram of this system. Each bucket supplied water to one arm of each Y-maze. The bucket located on the left supplied water to the right arm of each Y-maze and the right bucket supplied water to the left arms. The valve (Fig 2.3C), located at the start of the rubber tubing for each bucket, was used to turn on and off the water supply to the Y-mazes. This apparatus allowed us to run two separate trials simultaneously. A

close-up of the containers holding the water is shown in Figure 2.4. The entire apparatus can be seen in Figure 2.5.

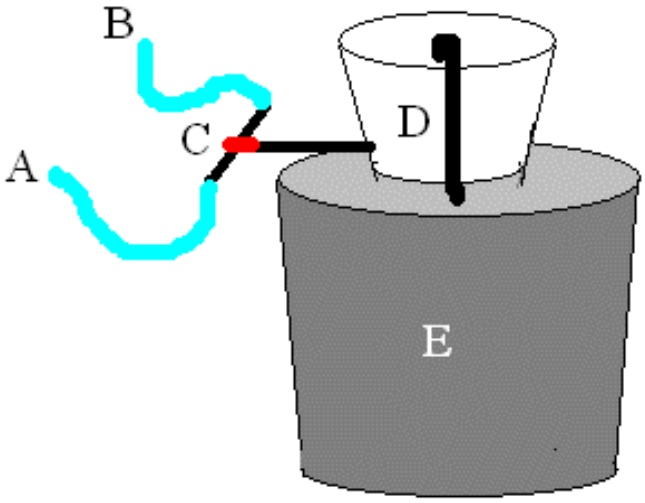


**Figure 2.3 Illustration of reservoir container and water flow to maze** *Two identical containers (E) contained the water used for the experiments. The water was pumped from this container to a smaller container above it (D). From there, the water passed through a valve (C) and into rubber tubing (A and B) which was connected to the arms of the Y-maze. Each bucket supplied water to one arm of each Y-maze. The valve (C) was used to turn on and off the water supply to the Y-mazes. This apparatus allowed us to run two separate trials simultaneously.*


**Figure 2.4 View of the water container part of the apparatus**


**Figure 2.5 An image of the entire apparatus**

## *2.3. Procedure*

Only sexually mature crayfish were collected for this experiment. All of the males were classified as Form I, possessing modified pleopods for sperm transfer, so they were determined to be sexually mature. The females were also classified as sexually mature because they were among the largest collected from the site, for this reason they were most likely sexually mature. Before the experiments were conducted, all of the non-virgin female and virgin female crayfish were tagged. Numbers were written on Tyvek paper with permanent marker and then glued to the carapace of each crayfish with superglue. The number of each crayfish along with its carapace and antenna length was recorded in addition to any abnormalities that were observed. Only those crayfish with two intact claws were used in the trials. When conducting our trials, we did not discriminate based on size.

Each trial required four liters of conditioned water: two liters of control water and two liters of experimental water. The control water was conditioned in a clean ten gallon aquarium. Four liters of water were added to the tank along with four clay pots and allowed to sit for twenty-four hours prior to the trial. The experimental water was also conditioned in a ten gallon aquarium containing four liters of water. Four crayfish of the same gender, either males or non-virgin females depending on the trial, were then placed in the tank along with four clay pots for shelter. The tank holding the experimental water was also allowed to sit for twenty-four hours prior to the trial. At this point the water was collected for use in the experiment and the crayfish were returned to their habitat tanks. Four different crayfish were then moved into the conditioning tanks. The same tank was used continuously for each type of conditioned water so not to cross contaminate the water.

Prior to the start of the trial, two liters of water were collected from each conditioning tank. Water was siphoned from the experimental tank to prevent disturbing the crayfish. The water was then placed into two 1 L glass jars to transport to the apparatus. Two focal female crayfish were removed from their

holding tank and brought to the apparatus as well. Their numbers were recorded along with the trial date and the gender being tested against.

Both of the 56.8L containers were filled to capacity with tap water. The crayfish were then placed in the stem of each Y-maze and the gate was lowered to prevent them from exploring all parts of the maze until they were acclimated to the water. The valves were then opened and the water was allowed to flow for five minutes to allow the crayfish to acclimate before the trial started. The buckets were then refilled to capacity. A coin was flipped to determine which bucket received the experimental water. The two liters of conditioned water were then added to each of the respective buckets. At this point, videorecording of the trial began.

A digital video camera was positioned on a tripod overlooking the Y-maze. At the beginning of each trial, we recorded the date, crayfish being tested, the experimental water type and arm through which each water type was flowing. The crayfish were released from the gate and allowed to explore the y shaped maze freely. The trial continued until all of the water had been pumped through the system; this took between seven and eight minutes. During the experiment, we did not lean over, stand above, or near the apparatus, so as not to startle the crayfish. Once the trial was complete the crayfish were returned to their habitat tanks and tap water was run through the apparatus to rinse out the experimental water. Each crayfish was used twice, once testing against male water and once against female water.

## *2.4. Data Analysis*

Once data had been collected each trial recording, which was 7-8 minutes in length, was analyzed. We recorded how long the crayfish was in each section of the Y-maze. The crayfish were determined to be in a section when their rostrum entered the section. The time spent in each area of the maze was quantified in seconds. We then calculated the difference between the time each crayfish spent in the experimental arm and the time spent in the control (experimental-control). The average was calculated for each treatment and the data were found to be non-

normally distributed. A SAS program (Appendix A) created by Gillette et al. (2000) was used to analyze the data for significance. For each treatment, the program multiplied each of the data points randomly by 1 or -1 to create artificial means. This was done 6000 times and the results were then compared to the actually mean of the data. A p-value was calculated as the percentage of the randomly generated means that were above, if the mean was positive, or below, if the mean was negative, the actual mean. Our significance criterion was set to $\alpha=0.05$. Each treatment type was tested separately and run through the SAS program three times to ensure that the results were reliable. We then averaged the three p-values for each treatment to get our final p-values.

# 3. Results

The data sets were analyzed in a number of ways. First, the time each crayfish spent in the control arm of the Y-maze was subtracted from the time they spent in the experimental arm. A positive value represents a crayfish that spent more time in the experimental arm, while a negative value represents more time spent in the control arm. Each treatment group was then averaged and these values were graphed, as shown in Figure 3.1. Microsoft Excel was used to determine the standard error, which is also shown in Figure 3.1.

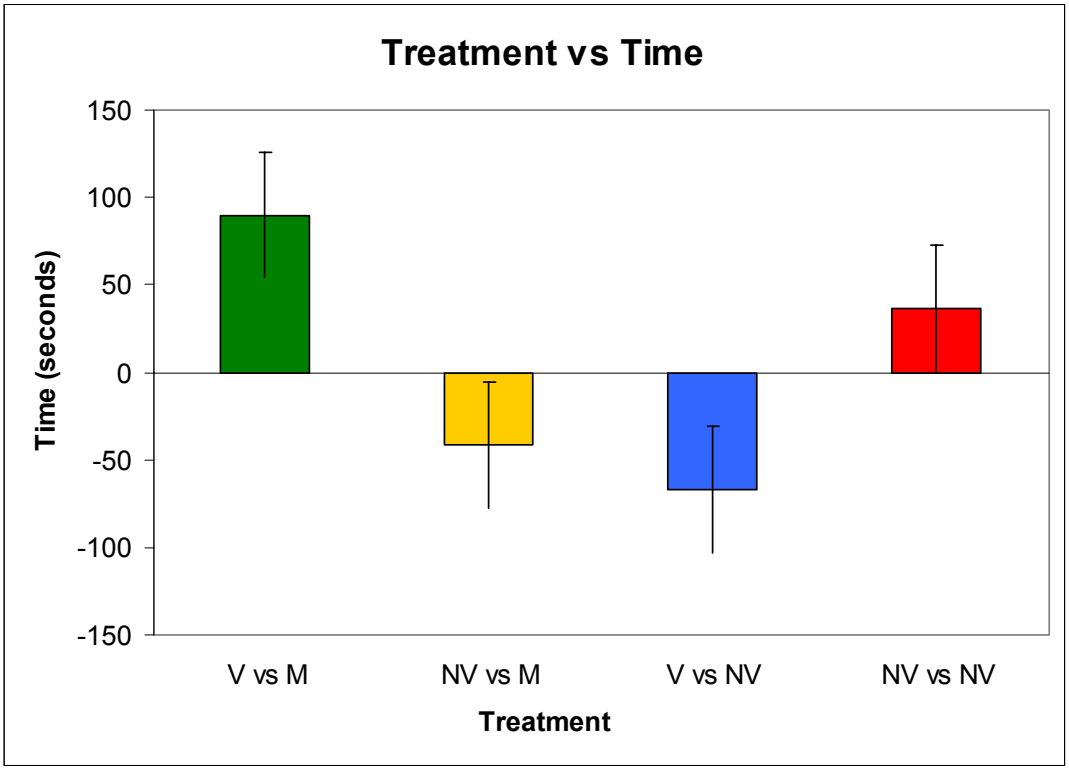


**Figure 3.1 Mean difference in time spent in treatment arm minus time spent in control arm for each treatment. Error bars are standard error.** *V vs M = virgin females tested against male-treated water; NV vs M = non-virgin females tested against male water); V vs NV = virgin females tested against non-virgin female water); NV vs NV = non-virgin females tested against non-virgin female water.*

It was found that the virgin females spent significantly more time in the experimental arm of the maze when they were being tested against male water, spending on average

about 90 seconds more in the experimental arm (Fig. 3.1; Table 3.2). When the virgin females were tested against non-familiar, non-virgin females, on average, they avoided the experimental arm. However, this difference was not statistically significant (Table 3.2), and they only spent about 65 seconds more on average in the control arm than in the experimental arm.

In another set of our trials, we tested non-virgin females against male experimental water. They preferred the control arm of the Y-maze over the experimental arm with the male conditioned water, but their preference was not statistically significant. On average, the non-virgin female crayfish spent 42 seconds more in the control arm. The averages and standard deviation can be seen in the blue bar in Figure 3.1. We also tested the non-virgin females against non-virgin female water. They had a slight preference towards the experimental arm. They spent an average of 37 seconds more in the experimental arm than in the control arm. The red bar in Figure 3.1 shows this along with the calculated standard deviation.

The only treatment with significant p-value was the virgin females being tested against the male conditioned water. All the p-values can be seen in Table 3.2.

**Table 3.2 Averaged p values for each treatment type**

| **Treatment** | **p value** |
|---|---|
| Virgins vs Male | 0.006167 |
| Virgins vs Non-virgin | 0.943333 |
| Non-virgin vs Male | 0.878333 |
| Non-virgin vs Non-virgin | 0.115333 |

While it was not statistically significant, it was interesting to note that each of the non-virgin females seemed to have a preference when it came to being attracted to or avoiding the non-virgin female experimental arm. The time spent for each individual crayfish can be seen in Figure 3.3. In the other trials, the female crayfish did not display the individual preference for one arm over the other. They all responded in a similar matter, whereas in Figure 3.3, some spent much more time than others in the control or experimental arm.

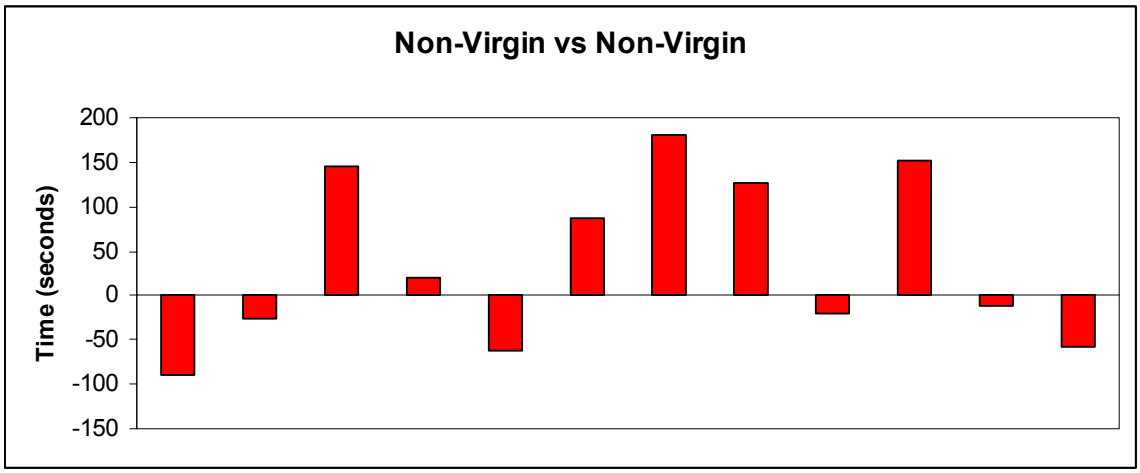


**Figure 3.3 Graph of mean difference in time spent in treatment (non-virgin female water) arm minus time spent in control arm for non-virgin females individually**

We also investigated the idea that the size of the crayfish may play a role in its reaction to the experimental water. To determine if it did, the length of each crayfish's carapace was plotted versus the difference in time spent in the experimental and control arm. The results can be seen in Figure 3.4. Using Excel, a line of best fit was added to each data set. All four data sets yielded lines that were nearly straight, and therefore there was no sign that size had any effect on crayfish responses to experimental water.

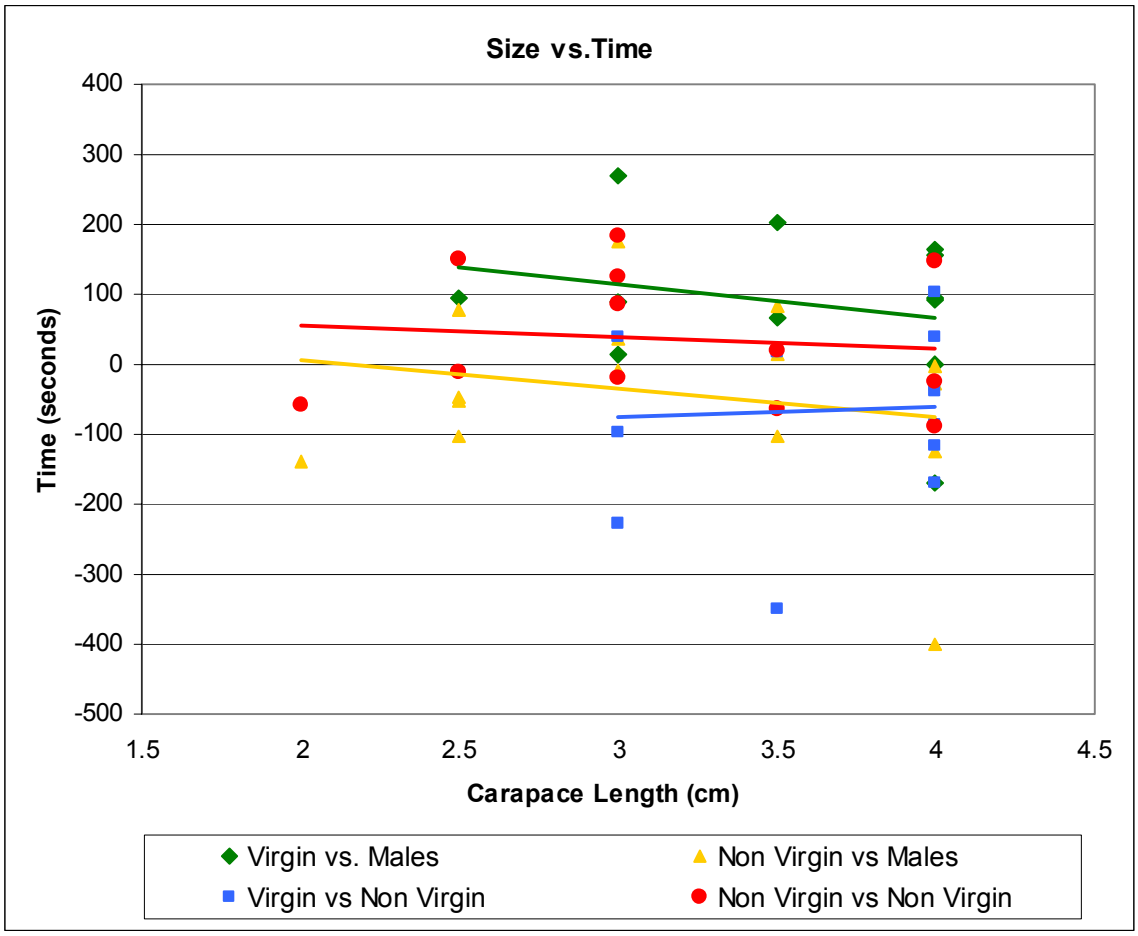


**Figure 3.4 Graph of crayfish carapace length versus the difference in time spent in the experimental and control arm**

# 4. Discussion

The virgin females preferred the water conditioned with males to the control water, and, through the SAS analysis, we found this data to be significant. This supports our hypothesis. We believe that this is probably due to the fact that the virgin females are looking for a mate by following the chemical signals of the males in the water. Interestingly enough, male crayfish were found to be attracted to virgin females when tested against control water in the same apparatus (Durgin, unpublished data).

When the virgin females were tested against non-virgin female conditioned water, they had a slight tendency to avoid the experimental arm. These data were not found to have a significant p-value. There are a couple reasons why the virgins would avoid or show no preference to the non-virgin female conditioned water. It is possible that the virgins would avoid the non-virgins because they were not familiar and they were avoiding a possibly aggressive situation. It is also possible that the crayfish are trying to minimize competition for mates. This may not be the case though, as data from another experiment suggests that non-virgin females give off a different chemical signal than virgins (Durgin, unpublished data). It has been shown that males can tell the difference between virgins and non-virgins, however it has yet to be tested whether or not females can detect a difference between virgins and non-virgins. We expect that the females will also be able to detect a difference between virgins and non-virgins. If males are more attracted to virgins, then female crayfish actively searching for a mate would benefit more from avoiding virgins than non-virgins as it decreases competition.

As stated in our results, the non-virgin females preferred the control arm to the experimental male conditioned water. We believe that this is due to the fact that the female crayfish have already mated. Since they have already mated, they are no longer interested in finding males to mate with. This is contrary to what has been found in other crayfish species (Walker et al, 2002; Galeotti et al., 2007). While our data shows that virgin females are more attracted to males than non-virgin females, it does not show conclusively whether they do in fact mate only once or multiple times. It is possible that *Orconectes virilis* does not practice polyandry like the other species, because the potential benefits (*e.g.,* more offspring or more fit offspring due to the competition between sperm) are outweighed by the costs (*e.g.,* injury from mating or competition, or

extra energy expended). It is also possible that once they have mated that season, they are no longer actively searching for a mate. However, if they do come across another male, they may choose to mate again. Male crayfish of this species were found to be attracted to virgin females, but they were also found to be attracted to non-virgin females to a lesser degree. Males probably prefer mating with virgin females because when they mate with non-virgin females, their sperm has to compete with the sperm that is already present. Whether or not this species practices polyandry, we believe that the females are aware of the fact that they have or have not mated and this plays an important role in their attraction to or repulsion from the males.

The non-virgin females also showed an overall preference to the experimental arm with the non-virgin female conditioned water; however the p-values show that this is not significant. We believe that they may have had this preference because they were familiar with the other non-virgin females as they were housed in the same tank. Also, we think that females may produce different pheromones once they have mated and therefore other crayfish can determine this. Therefore, the non-virgin females can tell that the other females have also mated so they are not competing for mates.

Closer examination of our data also shows that the non-virgin females each had their own very distinct preference for which arm they chose. Half heavily preferred the non-virgin female experimental arm and the other half preferred the control arm (refer to Figure 3.3). We think that this may show a dominance hierarchy between the female crayfish as they all lived together in the same tank. For example, if the female that was being tested was a subordinate of those females that were used to condition the treatment water, then she would be expected to avoid the treatment arm. Conversely, if the female being tested was dominant to those used for conditioning the treatment water, she would not be expected to avoid the treatment arm. We would have to do more experiments to determine the cause for these results.

As mentioned in our results, we decided to test to see if the size of the crayfish played a role in their attraction or repulsion towards the experimental water. We originally thought the smaller crayfish would be less attracted to the male experimental water, but we found no correlation between size and attraction or repulsion.

Further research could be done to determine if females mate multiple times. This could be done both by observing the crayfish mate and by collecting females carrying fertilized eggs to determine paternity. If it is found that *Orconectes virilis* is monogamous, further research could be done as to why this is the case with this species and not with others.

We concluded that our main hypothesis, that virgin females would be attracted to male conditioned water, was supported by our data. It was also found that non-virgin females showed no preference for male conditioned water. Our data is inconclusive as to whether or not *Orconectes virilis* practice polyandry because only virgin females were attracted to males, whereas the males were attracted to both virgin and non-virgin females. Further research into this subject in addition to our data would help determine the mating habits of this species.

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# 6. Appendices

## *6.1. **Appendix A:** A SAS program created by Gillette et al. (2000) used to analyze the data for significance. Shown here with sample data numbers.*

SAS Program

```
options ls=72 ps=60;
data one;
input diff;
datalines;
73
-49
-16
-313
-187
2
30
-10
38
56
-284
32
76
69
-57
59
184
-47
37
-45
10
117
82
36
-41
85
276
19
-37
-35
;

run;
```

```
%macro p;

data random; set one;
dummy=ranuni (-1);
run;

data two; set random;
if dummy<0.5 then diff=diff*-1;
run;

proc univariate data=two noprint;
var diff;
output out=three mean=meandiff;
run;

proc append base=combine data=three force;
run;

%mend p;

%macro m;
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%p
%mend m;

%macro g;
%m
```

```
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%m
%mend g;

%g
%g
%g
%g
%g
%g
%g
%g
%g
%g
%g
%g
%g
%g
%g
%g ;

data treat; set combine;
counter=1;
if meandiff>5 then counter=1;
if meandiff<5 then counter=2;
run;

proc freq data=treat;
tables counter;
run;
```