Effects of chemical communication on fighting and mating interactions in the crayfish *Faxonius virilis*

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Abstract

In many aquatic animals, chemical communication is a primary form of communication in which messages are inadvertently or intentionally transmitted between conspecifics. Chemical communication is often used to establish relationships in the context of antagonistic and mating encounters. We conducted two experiments to determine the impact of chemical communication in fighting interactions between two male *Faxonius virilis* crayfish as well as determine the impact of the “winner effect” in mating interactions with females. Through sex- and size-matched dyadic contests we tested the hypotheses that if crayfish are exposed to chemical signals from conspecifics prior to a contest, they would obtain information from those signals that may affect fighting dynamics in the future. We did not find a clear relationship between chemical exposure prior to a contest of male crayfish dyads and the behavioral outcomes of the fights. Dyadic encounters between size-matched reproductive male and female crayfish were also used to test the hypothesis that if a male crayfish wins in a previous fight with a conspecific male, then they are more likely to have mating success with females than males that lose a previous fight. From this experiment we found that the “winner effect” did not have an impact on mating interactions between male and female crayfish. However, we found a significant effect of average carapace length on the total duration of mating. Further research should investigate the relationship between factors that may influence fighting and mating outcomes of crayfish populations.

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Authorship

All authors contributed equally to this project.

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1.0 Introduction

Social behavior in crayfish has been the subject of a great deal of research regarding chemical communication. Prior research has focused on the interaction between chemical communication and social dominance in establishing a social structure. In this paper, we report on two different experiments. First, we report on the impact of chemical communication in the resolution of a contest between male-male crayfish dyads. Next, we report on the potential impact of female choice and the “winner” effect, both short and long term, on subsequent mating success of these male crayfish.

Crayfish are large invertebrates that belong to the phylum Arthropoda, class Crustacea, and the order Decapoda. They can be found in freshwater environments such as streams, ponds, lakes, marshes, and swamps. They come in a variety of sizes, shapes, and colors and the majority of species live in eastern North America. No matter what type of environment the crayfish live in, nearly all species use any type of shelter they can find for the majority of their lives (Crandall & Buhay, 2007). Rocks, logs, and underground burrows serve as shelter for crayfish to stay safe from predators and return to after eating, scavenging, or mating. Although they tend to live in high densities, crayfish do not form social groups. Instead, their social interactions are built upon dominance hierarchies. Crayfish mating season usually takes place during fall, but this may vary for some species (Longshaw & Stebbing, 2016). During their lifespan, male crayfish undergo alternations between two morphological forms. Form 1 indicates that the male is reproductively mature and Form 2 indicates that the male is reproductively immature (Hobbs, 1989). Reproductively mature males transfer sperm to the female’s sperm storage structure. However, fertilization and hatching of the eggs do not occur until the following spring.

1.1 Communication of Social Status in Aquatic Animals

Chemical communication is a primary form of communication used by many animals, including aquatic animals, in terms of habitats, food, mating, and competitiveness. Communicating with chemicals allows aquatic animals to inadvertently or intentionally convey a message from one animal to another. For example, a species of seabird uses chemical signals released when phytoplankton are attacked by zooplankton to locate food such as zooplankton, fish, and other birds (Nevitt, 2000). Another example can be found in female *Hapalogaster dentata,* also known as stone crabs, that use chemical signals to detect the size and dominance of male crabs as well as the sperm availability of the males in helping to choose a mate (Sato & Goshima, 2007). The American lobster, *Homarus americanus*, has been shown to use chemical signals to form social hierarchies, to find and choose mates, and to recognize conspecifics. Many other decapods, such as crayfish, also use chemical signals for similar purposes (Duffy & Thiel, 2007). Olfactory communication via odors released by crayfish is a major form of chemical communication and it has been found that naive crayfish who smell a dominant odor are more likely to act subordinately and lose fights while those who smell a subordinate odor are more likely to win encounters (Bergman & Moore, 2005).

While chemical communication is important in the development of social hierarchies and dominance structures in many species, there are other avenues of communication that can be used to establish social status. Visual cues play an important role in gaining insight into the status of another conspecific, which crayfish can use in subsequent fighting or mating interactions. Crayfish visually exposed to a fighting dyad prior to their own encounter with a naive crayfish were impacted in their interactions, as they initiated fewer fights, fought longer with the naive crayfish, and the fights were slower to escalate than crayfish in control treatments (Zulandt, Zundlant-Schneider, & Moore, 2008). It has also been found that visual stimuli from a crayfish of either sex increased the number of aggressive females, suggesting that visual stimuli are important for sex identification in female crayfish (Aquiloni, Massolo, & Gherardi, 2009). These various types of communications that crayfish use aid in interactions with one another and the development of social relationships.

Crayfish have an intricate social hierarchy that allows them to determine the status of other crayfish when engaging in a fight or mating interaction. Crayfish have been found to have multiple indicators of status, such as specific pheromones, postures, or behaviors. These indicators of status allow a crayfish to “size up” another crayfish that it is interacting with and determine whether that crayfish is higher or lower than itself within the social hierarchy. Such information gathering gives crayfish an advantage in knowing how to interact with one another. Aquiloni, Gonçalves, Inghilesi, & Gherardi (2012) conducted an experiment that consisted of two different treatments regarding visual and olfactory cues. They found that focal male crayfish that were allowed to eavesdrop visually and chemically on fighting conspecifics showed altered behavior in subsequent fights with the crayfish they observed. This indicated that the focal crayfish gained information from eavesdropping on the fight that could be used for future opponents. Additionally, Bergman, Kozlowski, Mcintyre, Huber, Daws, & Moore (2003) performed a two-part experiment initially revealing that there is a clear short-term winner effect in *O. rusticus.* The second part of the experiment showed that winning crayfish communicated this winner effect to male conspecifics in subsequent encounters through chemical signals.

While there is reliable evidence that chemical communication may play a part in forming social hierarchies, there is actually very little known about what types of messages are transmitted among crayfish via chemical communication. Therefore, we focused on the effect of chemical signals released by males prior to a fight with another male to discover what they can learn about each other’s social status through chemical cues alone. We hypothesized that if crayfish are exposed to chemical signals from conspecifics prior to a contest, they will obtain information from those cues that may affect fighting dynamics in the future.

1.2 Factors Influencing Mating Interactions

Mates are determined among animals via the reception of various social and biological cues through their senses, and these cues may include visual, auditory, olfactory signals, or some combination of such signals. In an investigation into the nature of chemical communication between male and female crayfish, Berry & Breithaupt (2010) found that female *Procambarus leniusculus* release urine that contains cues eliciting mating behavior. If female urine release was blocked, the male crayfish did not initiate mating. Acquistapace, Aquiloni, Hazlett and Gherardi (2002) found that males exposed to female odor and visual cues spent more time in locomotion and less time under their shelter, showing that males have the ability to recognize females through chemical and visual cues. It has also been found that male crayfish are able to distinguish between virgin and non-virgin females based on sex pheromones released in their urine (Durgin, Martin, Watkins & Mathews, 2008).

In a variety of different species, social interactions are impacted by the "winner" effect, in which winning a fight alters subsequent behavior in predictable ways (increasing the chance of winning another contest or having success at some other activity). The inverse of this is called the "loser" effect and applies to those who have lost an interaction. Teseo, Veerus, & Mery (2016) found evidence of this loser effect, where they observed that in fruit flies, loser males mated less than winners and control males. The winner effect is a major topic of study in the animal kingdom and its influence on interactions not only in fighting interactions but mating interactions as well. The winner effect has been determined to be instrumental in the development of dominance hierarchies as an extrinsic factor, which is independent of the physical skill of the organism (Dugatkin, 1997). While the impact of the winner effect has been researched extensively in the context of fighting and outcomes of future fights, its influence on mating is inconclusive, as some research claims that the winner effect does have an effect on mating, while other researchers have found that the winner effect does not affect mating interactions. Zeng, Zhou, & Zhu (2018) found that during the first two hours after fighting, winner male crickets had an increased reproductive fitness during pre-copulatory selection in comparison to loser crickets.

Alternatively, it was found that when female earwigs were allowed to choose between naive and winner or loser male earwigs, the females did not discriminate based on the males' recent fighting history, suggesting that recent fighting experience might not play as much of an important role in mating (van Lieshout, van Wilgenburg & Elgar, 2009). Additionally, in crayfish, it was found that females often gravitated towards dominant males by spending more time in close proximity to them as well as having more interactions with them than subordinate crayfish. However, this was only the case when the females had previously watched and smelled those crayfish, showing the capability of the female to recognize the winner as an individual and not a generic dominant male, which suggests that the winner effect was not a factor in these interactions (Aquiloni & Gherardi, 2010). These examples suggest that factors other than the winner effect might influence mating success, such as male superiority and female preference for certain males based on physical or situational factors.

The factors that influence why certain male and female crayfish mate with each other is a well-researched topic. However, our focus was to determine whether males and females are more likely to mate because of the winner effect or because there is a correlative relationship between factors that make males more competitive in fights that also make them more successful in mating. For example, males that win fights may be genetically superior to males that lose fights. We hypothesized that if a male crayfish wins in a fight with a conspecific male, then they are more likely to have mating success with females than males that lose a previous fight. Furthermore, the winner effect predicts that males who win a fight and mate immediately after will have higher mating success than males who win a fight and mate 1 week later.

2.0 Methodology

2.1 Crayfish Collection and Maintenance

Crayfish are often found in freshwater environments both in flowing systems such as rivers and streams, as well as standing bodies of water such as ponds, lakes, and marshes. Stream-dwelling crayfish often use shelter such as rocks, logs, and sticks close to shores in order to hide from predators and move in more open areas at night to find food (Longshaw & Stebbing, 2016). The crayfish collection sites used for the experiments were located in central Massachusetts, as seen in Figure 1.

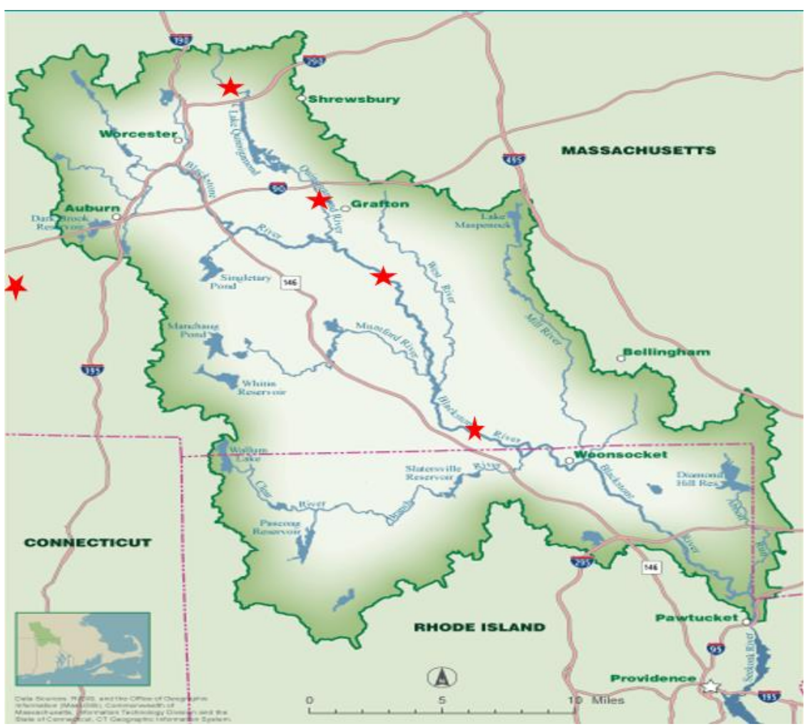

Figure 1: Map of the Blackstone River watershed. Sites are indicated by the red stars.
http://www.thebrwa.org/map.htm

We used *Faxonius virilis* because it is the most common species of crayfish in our area and is easier to collect in large quantities. We collected crayfish during September and October by hand and using small individual nets as well as by seining. We used seining in rocky parts of the collection sites, which entailed two members holding the ends of the seine and two members kicking rocks from the floor of the collection site so that any crayfish taking shelter under those rocks were moved downstream into the net.

We identified male and female crayfish and separated them by sex after they were caught. We identified males through gonopods, which are a modified set of swimmerets found on their underside that are enlarged and hardened. We identified females by a lack of enlarged gonopods and the presence of an annulus ventralis, which leads to the spermatheca. We returned all crayfish not missing any appendages to the greenhouse at Worcester Polytechnic Institute (WPI).

After each collection trip, we measured the length of each crayfish's right claw and carapace length using a manual caliper, measuring to the nearest tenth of a millimeter. We allocated each usable crayfish into a 35.6 × 20.3 × 12.4 cm plastic bin with pieces of white PVC tube as a shelter varying from 1.5in to 2in in diameter. We marked the tube with the crayfish ID number, sex, and the number of the site it was collected from. Each bin containing a crayfish was put into the greenhouse area at WPI so that the crayfish could be exposed to natural sunlight. Pieces of black foam were placed in between each bin so that the crayfish did not have the ability to see their neighbors, isolating them as much as possible. Crayfish were isolated in order to ensure there was no communication occurring among the crayfish before fighting or mating. This sensory communication could possibly allow crayfish to establish social hierarchy and change their behavior when fighting or mating before the fighting or mating process has even begun (Moore, 2005). Each bin was filled about halfway with tap water. Once the bins were filled with water, a plastic grid was placed over them and was weighed down with rocks to prevent escape. As we collected crayfish throughout the experimental period, they were isolated for a minimum of one week after they were processed and housed. We fed the crayfish half a rabbit food pellet twice a week and gave them a 50% water change twice a week.

2.2 Chemical Communication Experiment

In order to investigate the influence of prior chemical exposure on contests between male conspecifics, we carried out dyadic contests of Form 1 male crayfish in confined bins under three treatments. For Treatment 1, we transferred the paired males directly from their own bins to a contest bin. For Treatment 2, we swapped the crayfish into each other's bin one hour before the contest. By doing this, we were able to compare crayfish who had been exposed to one another's chemical cues (Treatment 2) to crayfish with no prior exposure to any conspecific cues (Treatment 1). For Treatment 3, we swapped the crayfish into a different pair of crayfish's bins that they would not encounter in the experiment an hour before the contest. Thus, crayfish were exposed to chemical cues of conspecific males, but not the individual males they would encounter in the contest. We used Treatment 3 to investigate whether simply receiving chemical cues of another crayfish would affect the individual's decisions in the upcoming contest. The goal of these treatments was to see if the behavior of crayfish that received Treatment 2 was any different from both the other treatments, hence inferring that crayfish learn something about each other just by receiving chemical cues of specific individuals. Table 1 below provides a summary of the three treatments used in the chemical communication experiment.

Table 1. The three treatments used in the chemical exposure experiment and the sample size of each.

	Description	**Sample Size (dyads)**
Treatment 1	No prior chemical exposure	34
Treatment 2	Chemical exposure of male they are going to encounter	17
Treatment 3	Chemical exposure of another random male that they will not encounter	27

We paired the crayfish for the fighting experiment by carapace length to minimize the potential effect of this variable on the outcome of the contest. We then assigned each pair of crayfish randomly to a treatment.

We fed the crayfish two days before their fighting day, in order to eliminate hunger as a potential factor in the encounter of the crayfish. One day before their contest, we removed each crayfish from its holding bin and labeled each with either an X or O on their dorsal carapace in silver permanent marker for identification purposes. We then placed them individually in a clean 35.6 × 20.3 × 12.4 cm bin, filled with fresh tap water and with the PVC tube from their holding bin. On the day of the contest, we swapped crayfish between bins for the pairs assigned to Treatments 2 and 3 and allowed them to remain in the other bin for 1 hour before placing them in the contest bins. Crayfish in Treatment 1 were not disturbed during this period.

The contests took place in larger bins (41.2 x 27.9 x 17.3 cm). A video camera was placed in such a way that three bins fit in the frame. We put black foam in between each of the bins to prevent the influence that could arise from the crayfish being able to see one another prior to the treatments. The quality of the footage was in HD to ensure that every interaction between the crayfish would be clear. Three-letter code words were written on duct tape and placed in the corner of the video frame in order to identify each bin and to ensure that video scoring was done blind to the treatment. We removed the crayfish from their holding bins and placed them in the larger bin simultaneously. After thirty minutes, we stopped the video camera and placed the crayfish back in their original bins for monitoring. Any replicates in which a crayfish died or molted within 3 days of the experiment were eliminated.

2.3 Mating Experiment

We tested the hypothesis that winners of fights would have more mating success than losers. Treatment 1 consisted of placing males and females together to mate immediately after the male-male fight and we predicted that winners in this treatment would take less time to initiate mating, have a longer first mating interaction, and have a longer total mating duration than winners in Treatment 2. Treatment 2 consisted of placing males and females together to mate 7 days after the male-male encounter and the winner effect predicts that winners in this treatment would take longer to initiate mating, have a shorter first mating interaction, and have a shorter total mating duration than winners in Treatment 1. This second experiment consisted of two treatments, as seen in Table 2.

Table 2. Description of both treatments in the mating experiment, along with the sample size in each.

	Description	**Sample Size**
Treatment 1	Female paired with male that immediately came from a contest with another male	19
Treatment 2	Female paired with male that had a contest with another male a week prior	35

We matched males and females by size using a carapace length of within 15% of each other. We then randomly assigned the male and female pair to Treatment 1 or Treatment 2. For Treatment 1, we removed males from the male-male contest bin and placed them into another 41.2 x 27.9 x 17.3 cm bin, half-filled with tap water, with their corresponding female. We put males that were assigned to Treatment 2 back in their original bins until their assigned mating date. A week after their contest, we put Treatment 2 crayfish in a 41.2 x 27.9 x 17.3 cm bin with their corresponding female, as was done with Treatment 1.

We placed four bins of the same size next to each other, forming a square on the ground. We put black foam in between each of the bins to prevent the influence that could arise from the crayfish being able to see one another prior to the treatments. We set up a video recorder above the four bins so that they were fully seen in one frame.

In order to identify the crayfish in analysis of the videos, we marked the female with a silver sharpie on the dorsal carapace as either an X or O, depending on what the male crayfish was marked with for its previous contest. Once the male and female were both in the bin, we turned the video camera on and set it to record on a non-HD setting since fine detail was not needed in order to determine if the crayfish were mating. After 3 hours, we stopped the video, we removed the crayfish from the bin and placed them back in their original bins. We monitored the crayfish used in both treatments for 3 days after the mating to ensure that they did not molt or die. If either of them molted or died, their replicate was removed from further analysis.

2.4 Scoring the Videos

The behavior codes that were used to score the fighting videos were modified from a study done by Bergman & Moore (2003). A description of the behavior codes and what they entail is found below in Table 3. Prior to formal scoring of the videos, about 10 minutes of video was viewed together as a group to standardize the scoring of the behaviors listed below. The videos were also scored blind to the experimental treatment.

The scoring of the fighting videos included the replicate name or number, which crayfish (X or O) completed an action, what the behavior was, the maximum intensity for fights, the start minutes and seconds of the behavior, the end minutes and seconds of the behavior, and a notes section.

Table 3. Behavior codes used to score the fighting videos from the chemical communication experiment.

Behavior Code #	**Behavior Code Description**
0	Ignore opponent with no response
1	Tail flip away from opponent or fast retreat
2	Slowly back away from opponent
3	Approach without threat display
4	Approach with threat display (meral spread and/or antenna whipping)
5	Chasing
6	Fighting with initial closed claw use
7	Fighting with active claw use by grabbing with open claws
8	Unrestrained fighting by grabbing

When a fight was recorded, a behavior code of 6 was given to indicate that a fight took place, but then a maximum intensity of either 6, 7, or 8 was also given next to the behavior code, representing the highest level of intensity that fight came to.

Most prior investigations refer to crayfish that have won a fight as dominant, but we reasoned that one 30-minute interaction was not sufficient in deeming a crayfish as dominant. Instead, we believed these crayfish should simply be considered winners of that encounter. Considering that our crayfish only participate in one contest, we will refer to them as winners and losers, instead of dominant and subordinate, in this paper.

To determine the winner crayfish of each contest, we decided on a threshold of 3:1 approaches to retreats, based on thresholds used in previous experiments (Driscoll, Kola, & Mathews, 2020; Fuxjager & Marler, 2010). This ratio meant that in order for a crayfish to be the winner of a contest they needed to have a ratio of at least three approaches towards the other crayfish to no more than one retreat away from the other crayfish. Also, the other crayfish had to participate in some manner so that both crayfish were making decisions. In other words, if one of the crayfish had zero approaches and zero retreats, then a winner could not be determined for that replicate. Interestingly, while scoring the videos we discovered several accounts of male crayfish from the chemical communication experiments mating with one another. We defined male-male mating attempts as when one male displayed mating behavior towards the other male, such as getting on top of the other crayfish, flipping them over, and pinning their outstretched claws, which is similar to behavior males direct towards females during initiation of mating.

Videos from the mating experiment were scored by noting the replicate number, the start minutes and seconds of mating, and the end minutes and seconds of mating for each mating incidence. After we scored all the fighting and mating videos, we created two separate analysis sheets for each experiment to make it easier for analysis in SPSS. The fighting analysis sheet included the treatment number, the replicate name, the crayfish IDs, the carapace length of each crayfish, whether there was a winner of the fight, whether the crayfish were used in mating, the total number of fights, the total duration of fights, the time until the first fight, the total number of approaches and retreats, and the total time until the threshold was met for determining a winner and loser. The mating analysis spreadsheet included the treatment number, replicate number, crayfish ID, whether mating occurred or not, the time before the first mating, the duration of the first mating, the total duration of mating, and whether the male won or lost its previous fight with another male. We then used these spreadsheets to do further analysis in SPSS.

2.5 Statistical Analyses

We did statistical analysis of the data collected in IBM SPSS. We performed tests of conformance to the normal distribution and homogeneity of variances for all variables using the Shapiro-Wilks test and Levene’s test, respectively. All of the variables met the assumption of equality of variances, while none were normally distributed. We attempted transforming the data using logarithmic and square root transformations, however neither of these transformations resulted in normally distributed data. We moved forward with parametric analysis of the non-normal data. While this assumption could invoke a Type I error, ANOVAs are tolerant enough with our sample size to allow us to move forward despite our moderate violation of normality (Blanca et al., 2017; Rheinheimer & Penfield, 2001).

The tests used for the fighting analysis of the chemical communication experiment were univariate analyses of covariance (ANCOVA), as there was one nominal independent variable (treatment number) with a covariate of carapace length (averaged between the paired interacting crayfish), and one measurement dependent variable being tested. Separate two-way ANCOVAs were run for the dependent variables of total duration of fighting and the time until the threshold was met. Additionally, we performed a Fisher's exact test using a spreadsheet provided by the Handbook of Biological Statistics (McDonald, 2014) to determine the proportion of male crayfish in which we could determine a winner from fights in each of the three treatments. We also performed a Fisher's exact test to determine there was a difference in the occurrence of male-male mating among Treatments 1, 2, and 3 in the chemical communication experiment.

We performed three two-way ANCOVAs for the mating experiment analyses because there were two nominal independent variables (treatment number and winner/loser status) and one measurement dependent variable (time until initial mating, duration of initial mating, total duration of mating), as well as a covariate of average carapace length. We also conducted a Pearson correlation analysis between the two variables in order to determine where there was a difference. For these tests, the males that did not mate during the mating experiment were taken out of this analysis, as to not skew the data into making it seem like the males mated immediately when in reality they did not mate at all. This left 22 individual males for analysis for these three variables. Finally, for the mating experiment, we performed a Fisher's exact test to determine if there was a difference in the proportion of winning and losing males that mated for each treatment. We saved the output charts of all tests done and created bar graphs to represent all the data.

3.0 Results

3.1 Chemical Communication Experiment Results

In the first experiment, there were a total of 77 replicates (n=33 for Treatment 1, n=17 for Treatment 2, n=27 for Treatment 3), with no replicates being removed due to death or molting within 3 days after the trial. For this experiment there were only 41 replicates (n=20 for Treatment 1, n=5 for Treatment 2, n=16 for Treatment 3) in which we could determine a winner and loser. A Fisher's exact test showed that the proportions of replicates in which a winner was determined or not was not significantly different between the three treatments (p=0.093).

We did not find evidence that chemical exposure or lack thereof from the different treatments had any significant effect on the interaction or outcome of the male-male crayfish contests. We found no significant effect of the treatment on the time until the threshold for assigning winner and loser status was met ($F[2,37]=0.126$, $p=0.882$; Figure 2). There was also not a significant effect of the average carapace length of the paired crayfish on the time until the threshold was met ($F[1,37]=0.356$, $p=0.554$). We found no significant effect of the fighting treatments on the total duration of fighting that took place ($F[2,73]=0.664$, $p=0.518$; Figure 3). Additionally, the average carapace length between the fighting dyads did not have a significant effect on the total duration of fighting ($F[1,73]=1.775$, $p=0.187$).

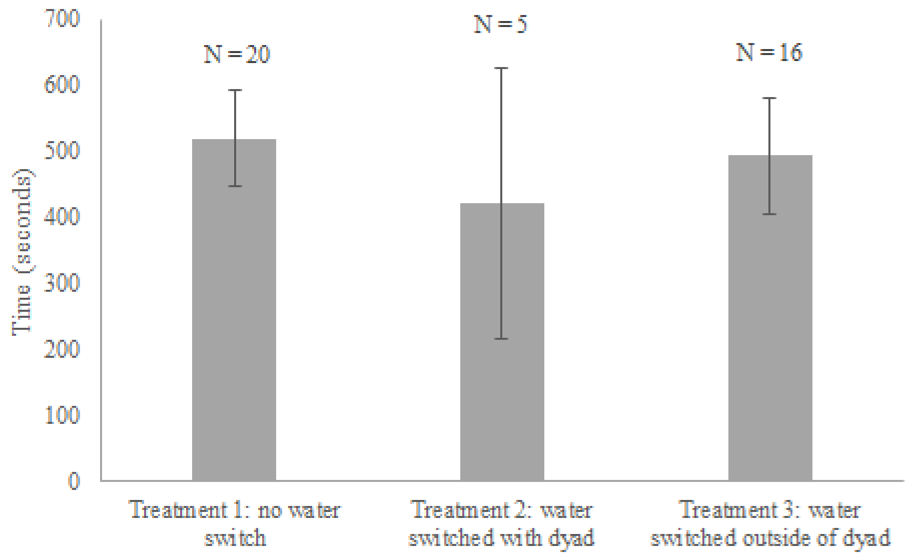

Figure 2. Average time until contest assessment threshold met for crayfish dyads in the three treatments. Error bars show standard error. Numbers above the errors bars indicate sample size.

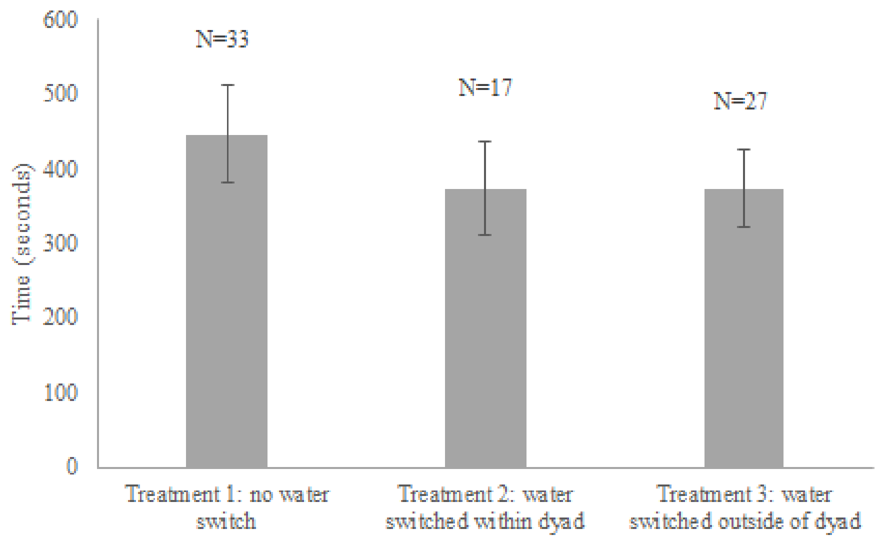

Figure 3. Average time spent fighting for crayfish dyads in the three treatments. Error bars show standard error. Numbers over the bars indicate sample size.

From the chemical communication experiment, there were 9 male-male mating attempts, in which one male displayed mating behavior, such as getting on top of the other crayfish, flipping them over, and pinning their claws above their head. A Fisher's exact test revealed that among the proportion of male-male mating versus no male-male mating, there was no significant difference among the three treatments (p=0.180; Figure 4). Table 4 displays a summary of the tests and predictions made regarding the chemical communication experiment.

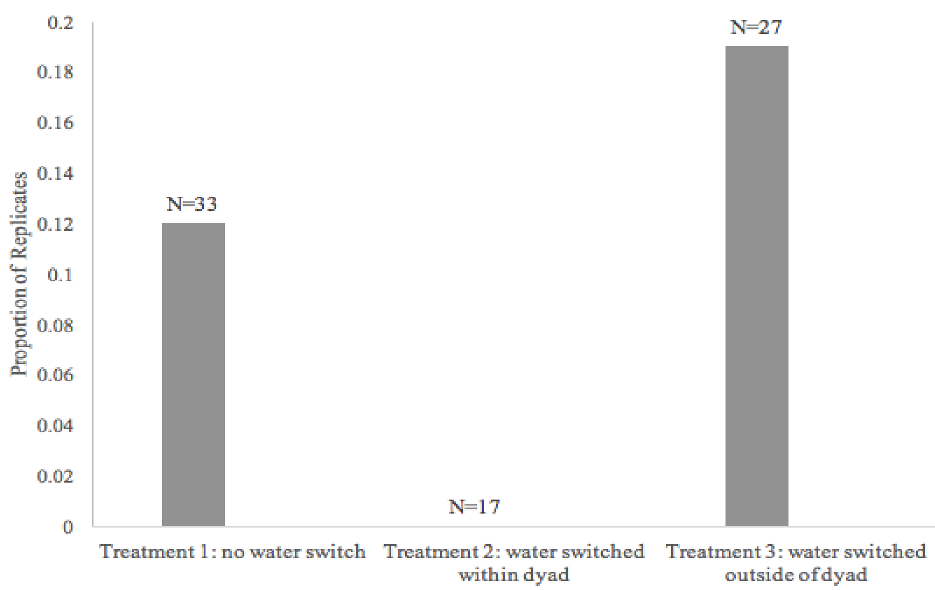

Figure 4. Proportion of replicates in each treatment in which male-male mating occurred or not. Numbers over the bars indicate sample size.

Table 4. Predictions tested in SPSS, along with the rationales and results from the chemical communication experiment types.

Test	**Variables**	**Predictions**	**Rationale**	**Result**
ANCOVA	Time until threshold met (seconds) Treatment #	Crayfish fighting in Treatment 2 will establish their relationship quicker than other treatments. Crayfish fighting in Treatment 1 will take the longest to establish a relationship. Crayfish fighting in Treatment 3 will take either the same amount of time as Treatment 1 or longer, to establish a relationship.	Previous research shows evidence of chemical communication between crayfish. Treatment 2 is the one treatment that crayfish can gain information prior to physically encountering their opponent, hence making it easier for them to establish a relationship. Crayfish in Treatment 3 will take longer than crayfish in Treatment 1 because they are swapped with a crayfish dyad that they are not encountering in the contest, therefore they may be confused and need more time to readjust the information they may or may not have received prior.	Unsupported
ANCOVA	Total duration of fighting Treatment #	Males in Treatment 1 will fight longer than the other two treatments. Males in Treatment 2 will fight for the least amount of time between the three treatments. Males in Treatment 3 will fight longer than males from Treatment 2, but shorter than males from Treatment 1.	Previous research shows evidence of chemical communication between crayfish. Treatment 2 is the one treatment that crayfish can gain information before physically encountering their opponent, hence making it easier for them to establish a relationship. Crayfish in Treatment 3 will take longer than crayfish in Treatment 1 because they are swapped with a crayfish dyad that they are not encountering in the contest, therefore they may be confused and need more time to readjust the information they may or may not have received prior, causing there to be more time spent fighting.	Unsupported
Fisher's exact test	Winner assignment Treatment #	Treatment 2 will have more winners assigned than the other two treatments. Treatment 1 will have the least winners assigned of the three treatments.	Since in Treatment 2 crayfish are allowed to gather chemical information from each other before physically coming into contact, it will be easier for them to establish relationships in the 30 min they fight. Crayfish in Treatment 1 have no prior exposure, so they may not provide enough evidence of a winner or loser in the specific timeframe, leading to the least number of winner/loser assignments of the three treatments. Crayfish in Treatment 3 will have more winners assigned than Treatment 1, but less than Treatment 2 because they had prior exposure, but to a random dyad of crayfish which will cause them to take longer to establish a winner or loser but may be able to do so based on having some form of prior chemical exposure.	Unsupported

Fisher's exact test	Male-male mating Treatment #	We did not expect to witness any male-male mating over the course of this experiment and thus did not develop a prediction.	N/A	N/A

3.2 Mating Experiment Results

For the mating experiment, there were a total of 55 replicates and one was removed due to death within 3 days after the trial, leaving 54 replicates (n=19 for Treatment 1, n=35 for Treatment 2). We were only able to assign winner and loser status to 17 replicates for both treatments, so this left us with 34 individual crayfish for the mating experiment. However, of these 34, only 22 males mated with a female which constituted our sample size for the analyses.

Analysis of main effects showed there was no significant effect of either winner and loser status ($F[1,17]=0.653$, $p=0.430$) or treatment number ($F[1,17]=2.553$, $p=0.129$; Figure 5) on the time until the initial mating occurred. There was also no significant effect of carapace length of the crayfish pair on the time until the initial mating ($F[1,17]=2.096$, $p=0.166$). There was not a statistically significant interaction between the winner and loser status and treatment number on the time until the initial mating ($F[1,17]=0.008$, $p=0.931$).

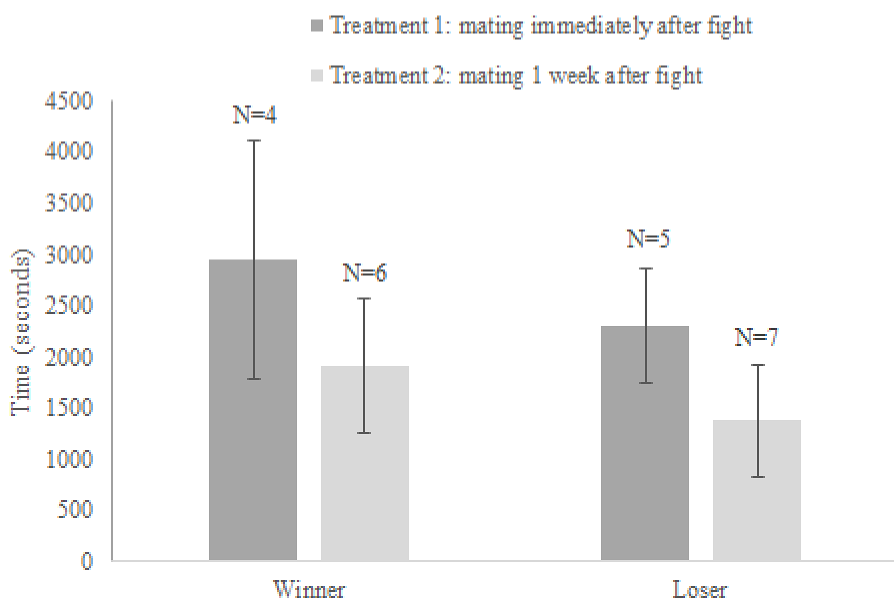

Figure 5. Time until initial mating by the male's winner or loser status in both treatments. Error bars show standard error. Numbers over the bars indicate sample size.

Simple main effect analysis showed there was no significant effect of either winner and loser status ($F[1,17]=0.074$, $p=0.788$) or treatment number ($F[1,17]=0.32$, $p=0.860$; Figure 6) on the duration of the initial mating. There was also no significant effect of average carapace length on the duration of the initial mating ($F[1,17]=0.265$, $p=0.613$). The interaction between winner/loser status and treatment number also had no significant effect on the duration of the initial mating ($F[1,17]=0.162$, $p=0.692$).

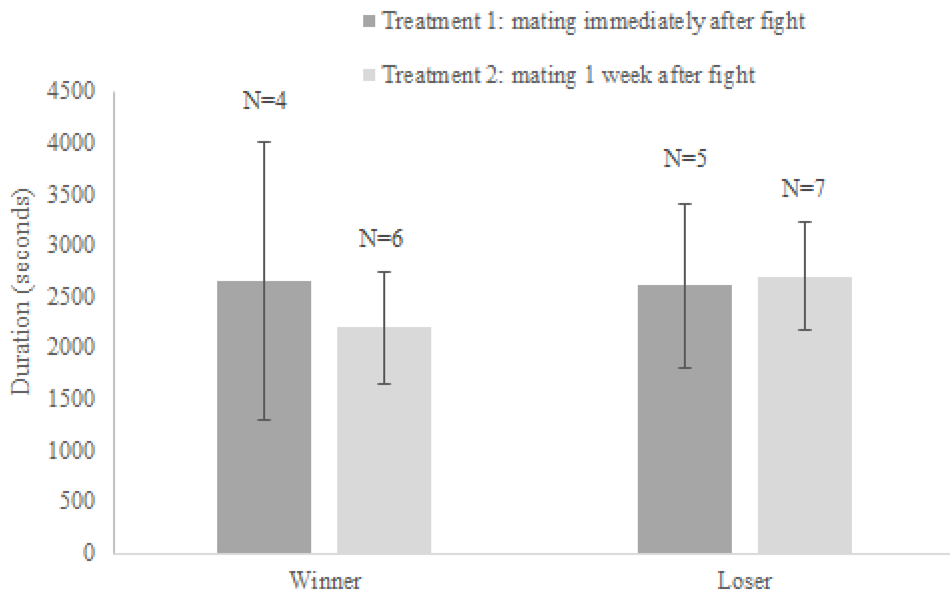

Figure 6. Duration of initial mating by the male's winner or loser status in both treatments. Error bars show standard error. Numbers over the bars indicate sample size.

A separate analysis showed there was no significant effect of either winner and loser status (*F*[1,17]=0.756, *p*=0.397) or treatment number (*F*[1,17]=1.135, *p*=0.302; Figure 7) on the total duration of mating. There was a significant effect of the average carapace length of the crayfish mating dyad on the total duration of mating (*F*[1,17]=9.373, *p*=0.007). A correlation analysis showed that there was a significant negative correlation between carapace length and the total duration of mating (*r*=-0.606, *p*=0.003; Figure 8).There was not a statistically significant interaction between the winner and loser status and treatment number on the total duration of mating (*F*[1,17]=0.597, *p*=0.450).

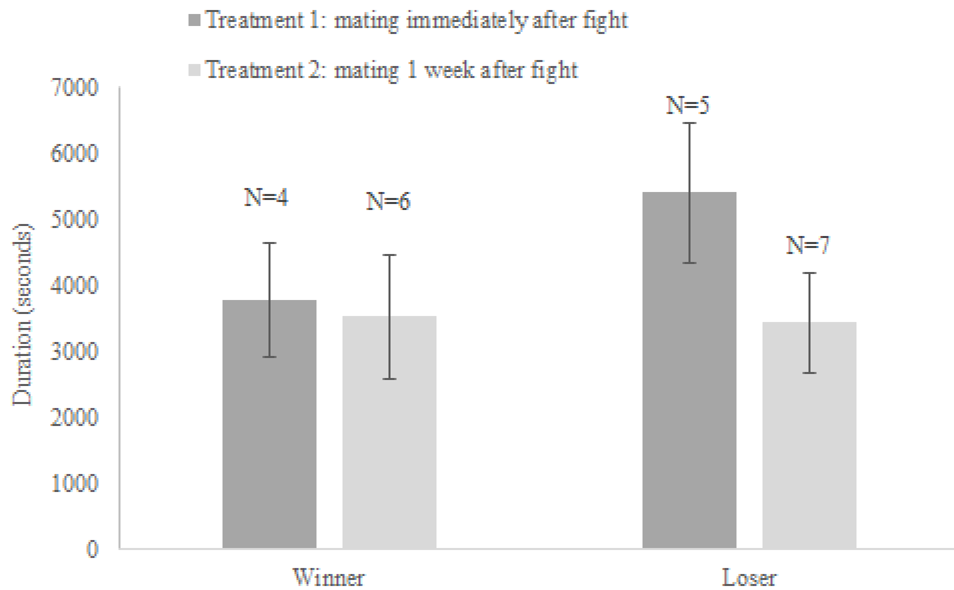

Figure 7. Total duration of mating by the male's winner or loser status in both treatments. Error bars show standard error. Numbers over the bars indicate sample size.

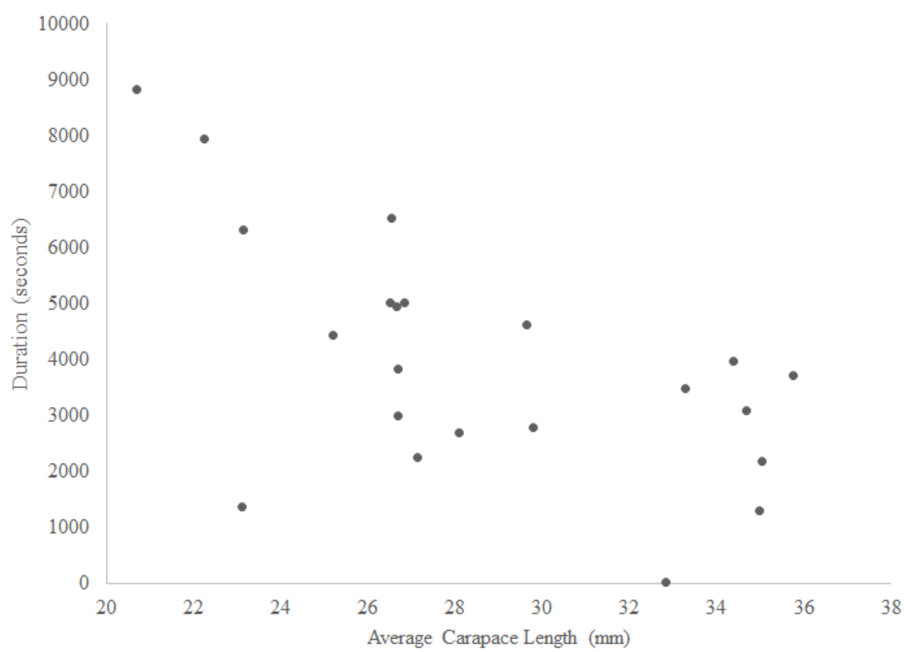

Figure 8. Total duration of mating versus average carapace length. N=22

There was no evidence in either treatment supporting that a male was more likely to mate after winning a fight than after losing a fight. A Fisher's exact test (Figure 9) revealed that the proportion of winning and losing males that mated were not significantly different in either Treatment 1 (p=1.0) or Treatment 2 (p=0.68). Table 5 displays a summary of the tests and predictions made regarding the mating experiment.

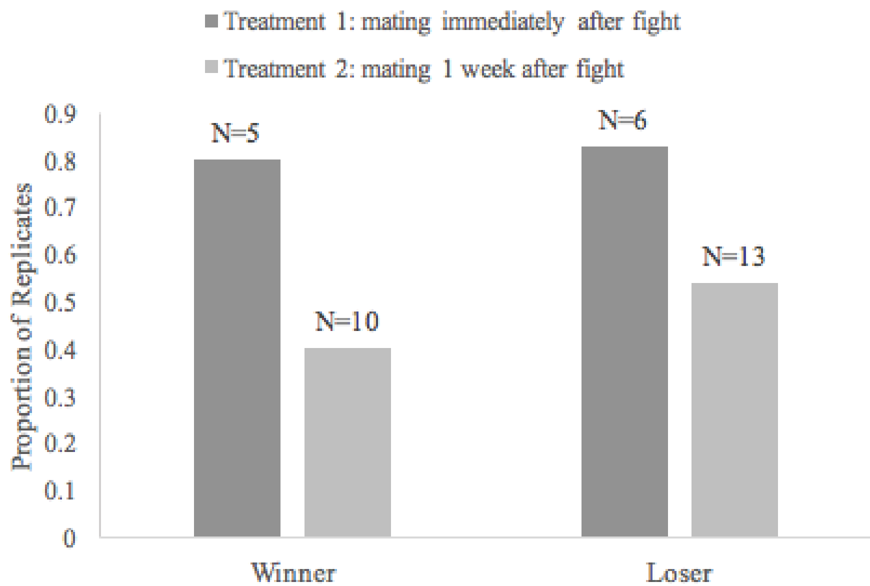

Figure 9. Proportions of winning and losing males that mated in Treatment 1 (mating immediately after fight) and Treatment 2 (mating 1 week after fight). Numbers over the bars indicate sample size.

Table 5. Predictions tested in SPSS, along with the rationales and results from the mating experiment.

Test	Variables	Predictions	Rationale	Result
Two-way ANCOVA	Time until initial mating Winner/loser Treatment #	Males that win a fight and mate immediately after the fight will take less time to start mating than males that win a fight and mate 1 week after.	Previous research shows evidence of a short term “winner” effect and males in Treatment 1 are more inclined to initiate mating due to this “high” state.	Unsupported
Two-way ANCOVA	Duration of initial mating Winner/loser Treatment #	Males that win a fight and mate immediately after the fight will mate longer in the initial mating session than males that win a fight and mate 1 week after.	Previous research shows evidence of a short term “winner” effect and males in Treatment 1 are more inclined to mate for a longer time due to this “high” state.	Unsupported
Two-way ANCOVA	Duration of total mating Winner/loser Treatment #	Males that win a fight and mate immediately after will mate for a longer total duration than males that win a fight and mate 1 week after.	Previous research shows evidence of a short term “winner” effect and males in Treatment 1 are more inclined to mate for a longer time due to this “high” state.	Unsupported
Pearson Correlation Analysis	Duration of total mating Average Carapace Length	We did not expect to see a significant correlation between duration of total mating and average carapace length.	N/A	N/A

Fisher’s exact test	Winning/losing males that mated Treatment #	In both Treatment 1 and 2, there will be more males that win a fight and mate in comparison to the males that lose and mate from this same treatment. There will be more males that mate regardless of winner loser status from Treatment 1 than from Treatment 2.	Previous research shows evidence of a “winner” effect and “winner” males are more inclined to mate due to this “high” state than “loser” ones. Research also shows that there is not much of a difference in crayfish who win a fight and mate immediately after a fight versus crayfish who win a fight and mate later on.	Unsupported

4.0 Discussion

4.1 Chemical Communication Experiment Discussion

Chemical communication is one important mode that crayfish, among other animals, use in social interactions to develop and maintain social hierarchies and dominance structures (Berry & Breithaupt, 2010). The social hierarchy that crayfish develop can influence behavior in interactions with one another. The focus of our experiment was to explore whether or not prior exposure through chemical communication will alter subsequent fighting interactions. We hypothesized that if crayfish are exposed to chemical signals from conspecifics prior to a contest, they will obtain information from those cues that may affect fighting dynamics in the future. However, our hypothesis was not supported, as we found no significant difference among Treatments 1, 2, 3 in any element of behavior that we compared.

While the data regarding how long it took for a threshold to be met in each contest (Figure 2) was not found to be significant, it did follow the trend that we hypothesized, in that dyads in Treatment 2 met the threshold the fastest, and Treatment 3 dyads met the threshold slightly faster than Treatment 1. This was what we expected to see due to the fact that Treatment 2 dyads had already come in contact with each other's chemical signals before the fight and because Treatment 1 dyads had not been exposed to the chemicals of any other crayfish we assumed they would take the longest to meet the threshold.

On the other hand, the data regarding the average total amount of time fighting in each contest (Figure 3) did not follow the trend that we originally expected. It was predicted that Treatment 2 dyads would have the shortest amount of total time spent fighting. We found that Treatments 2 and 3 had nearly the same amount of average total time spent fighting. It was clear that Treatment 1 had the greatest average total amount of time spent fighting, which fit our prediction. We predicted that Treatment 2 dyads would spend the shortest amount of time fighting because they would be able to establish a relationship faster than the other treatments due to the fact that they had already been exposed to each other’s chemicals.

The outcome of fighting interactions could have been affected by some of the following factors. It is possible that recording the contests for 30 minutes is not enough time to be able to capture the entire scope of the crayfish fighting interactions. Perhaps if the recording time had been extended to 45 minutes or 1 hour, more dyads could have met the threshold for determining winner or loser statuses. Also, the ethogram that was used for the fights to help analyze behavior focuses on easily distinguishable crayfish behaviors and is not a comprehensive guide to understanding how the crayfish are behaving. As with most experiments, especially with live animal subjects, a larger sample size is a major factor that shapes the results of the experiment. With a larger sample size we could have potentially had some significant results. With behavioral data, there can tend to be high variability, thus making it more difficult to detect any effects that may be happening unless there is a large sample size. It is also possible that the size of the bins for the fighting interactions were restrictive for the natural behavior of the crayfish, seeing as they are used to rivers and streams and having much more open area to interact or not interact with other crayfish. A proposed change for future experiments would be to have bin sizes that are more proportional to the size of the crayfish to allow for space that more closely mimics the amount of space they have in the wild rather than subjecting larger crayfish to the same space as crayfish that are half their size.

Overall, our data does not demonstrate a clear relationship between exposure prior to a contest between male crayfish dyads and behavioral interactions or outcomes of that contest. Our data suggests that prior chemical communication does not affect behavior in fighting interactions. This could mean that chemical communication may not be as important as was previously thought, or that there are other factors that may also contribute to social interactions and behaviors between crayfish. Previous research exploring the nature of crayfish interactions and behaviors in antagonistic behavior have found olfaction, vision, and touch (Callaghan, Dew, Weisbord, & Pyle, 2012), as well as chemical exposure through odors (Schneider, Schneider, & Moore, 1999) and urine (Bergman, Martin, & Moore, 2005) have significant effects on subsequent interactions between crayfish. It is possible that chemical communication alone is not sufficient for crayfish in fighting interactions and may need to include different modes of communication, such as sight, olfaction, and urine output in order to see a significant difference between treatments. Communication, especially chemical communication, is an important way in which crayfish, among other animals, navigate daily interactions regarding habitats, food (Nevitt, 2000), mating (Sato & Goshima, 2007; Duffy & Thiel, 2007), and competitiveness (Sato & Goshima, 2007; Duffy & Thiel, 2007). Therefore, it would be insightful for future large-scale experiments to look further into what, if any, specific information is communicated chemically between crayfish and how that may affect subsequent interactions with each other.

Most prior research did not focus on the total fighting time in a male-male interaction and the time until a threshold was met in order to deduce a winner or loser status. This is due to the fact that the threshold in these other papers consists of only one interaction between the dyads and their relationship is considered established at that point. The winner is the crayfish that first approached and the loser is the crayfish that first retreated in response to that approach. Prior experiments in which male crayfish fought did not analyze the impact of chemical communication on different aspects of the encounter such as total fighting time and time until threshold met. While our findings did not indicate any significant impact of prior chemical communication before fighting and the lengths of certain fighting parameters, we did see trends based on prior predictions.

We recorded 9 total instances of male-male matings across all of the fights for the three treatments. There were 4 fights in Treatment 1 in which we saw male-male mating and 5 fights in Treatment 3 in which we saw male-male mating. These instances of male-male mating were a surprising discovery and it is not fully understood why they occurred. Interestingly, there were no cases of male-male mating interactions in Treatment 2, in which the crayfish were swapped in each other’s bins before the contest. Further research will be required to investigate the function, if any, of these same-sex sexual interactions, and to determine if chemical communication is involved in mediating such interactions.

4.2 Mating Experiment Discussion

There are many potential factors that can influence mating interactions between crayfish. Some of these include chemical communication, the “winner” effect, female choice, or a relationship between more competitive crayfish being more successful in mating interactions. We hypothesized that male crayfish that win a fight with a conspecific male would have more mating success with females than male crayfish that lose a previous fight, which was overall not supported by our data. In addition, we investigated the impact that the winner effect may have on mating interactions, which predicts that males who win a fight and mate immediately after will have more mating success than males who win a fight and mate 1 week later. However, this prediction was not supported by our data.

Previous research has shown conflicting evidence of the influence that the winner effect has on mating interactions (Zeng, Zhou, & Zhu, 2018; van Lieshout, van Wilgenburg & Elgar, 2009). We found no evidence that the winner effect or other potential factors had a significant impact on mating success. We predicted that males that win a fight and mate immediately after the fight (Treatment 1) will mate longer in the initial mating session (Figure 6) than males that win a fight and mate 1 week after (Treatment 2). Although we found no significant difference, our data regarding the duration of the initial mating follows the pattern we predicted, showing that Treatment 1 winners and losers mated for longer in the initial mating session than winners and losers in Treatment 2.

Our predictions for three of our tests (total duration of mating, the time until the initial mate, and the proportion of winning and losing males that mated in each treatment), as seen in Table 5, did not follow the trend we predicted. We did not find any significant difference in any of the data analyzed for these variables. Specifically, we did not find that either winner or loser status or the treatment had any clear effect on whether mating occurred, how long it took to begin, or how long it lasted. However, we found that the carapace length of the crayfish had a significant effect on the total duration of mating. Figure 8 shows that larger crayfish dyads mated for a shorter duration than smaller crayfish dyads. Other research on crustaceans that have investigated the relationship between body size and the duration of copulation have found that there was not necessarily a significant relationship between the two variables (Jivoff, 1997). A reason as to why crayfish with a smaller carapace length may mate for a longer total duration could be because smaller crayfish have more difficulty establishing mounting positions and in sperm delivery (Jinbo, Sugiyama, Murakami, & Hamasaki, 2017) and therefore need more copulation time to mate successfully. Further research should be done to investigate the duration of mating in crayfish because there are other factors that may influence mating duration such as chelae size (Snedden, 1990), familiarity (Singh, Mishra, & Omkar, 2019), semen capacity (Helinski & Harrington, 2011) and cost of sperm production (Ward & Simmons, 1991).

Finally, we expected that within both Treatment 1 and 2, there would be more males that won a fight and mated in comparison to the number of males that lost and mated (Figure 9), but our data did not support this, and actually we saw a slightly larger proportion of losers mating than winners in Treatment 2. We also predicted that there would be more males that mated regardless of winner or loser status from Treatment 1 than from Treatment 2, and while our data did follow this predicted direction, there was no significance associated with this data. This experiment may have been subject to similar limitations as the prior experiment in terms of sample size and potential confounding effects of bin size.

The data from the mating experiment does not say much about the winner effect or other factors that may influence mating interactions and the relationship between them. There have been studies that find the winner effect does influence mating interactions with males that win a fight mating more than males that lose a fight (Zeng, Zhou, & Zhu, 2018), but there have also been studies that have found that the winner effect does not influence mating interactions (van Lieshout, van Wilgenburg & Elgar, 2009). Specifically, our data does not demonstrate that the winner effect influences mating interactions, which raises the question: what other factors are at play that influence mating behavior? Other studies have found that female crayfish tend to prefer males that are familiar to them over unfamiliar males (Kubec, Kouba, Kozak, & Buric, 2019) as well as females being more attracted to males that are of a larger size, have two chelipeds, and have ownership of burrows. (Villanelli & Gherardi, 1998). Mates in many animals are determined through various social and biological cues. Understanding the mechanisms and the reasons why crayfish choose mates may provide further insight into the social structures of crayfish, especially between males and females. Further research is needed to distinguish between the factors influencing mating success in crayfish, such as the winner effect, female choice, or genetic superiority associated with mating success in males.

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