THE IMPACT OF CHEMICAL CUE RECEPTION DURING AGONISTIC INTERACTIONS IN FEMALE CRAYFISH

Kelly Bryant

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Committee:

Paul A. Moore, Committee Chair

Daniel Pavuk

Rachelle Belanger

ABSTRACT

Paul A. Moore, Committee Chair

Agonistic interactions between individuals are influenced by a variety of complex factors both internal and external. Due to the complex nature of interactions, it can be difficult to determine the specific driving factors that influence the outcome of agonistic interactions. In many species, physical and chemical signals are utilized to deliver specific cues to potential opponents and mitigate interactions. In aquatic systems chemical signals are often used and designed to be carried by the flow of the water to deliver ranged information. This information can include status, sexual availability, aggression, and other important cues that may not be discernable in the water column through other channels. Crustaceans are a well-known group for modeling dominance hierarchies due to their overt demonstrations for dominance and repetition of agonistic behaviors over time.

The goal of this research was to investigate the role that chemical cue reception plays in determining dominance in agonistic interactions in female crayfish. To accomplish this, we generated groups of individuals and grouped them by size, form, and species to receive either the control or ablation treatment. Chemosensory ablation removed the animal's ability to detect chemical signals with their antennules through an extensive lesioning process which lysed the cells on that sensory organ. We discovered that lesioning of the antennules resulted in changes in duration and level of escalation of agonistic interactions among the crayfish species tested. Additionally, the size of the crayfish was a contributing factor to the duration and intensity of the interaction. Lesioned crayfish of larger size spend longer at low intensity agonistic behavior, likely due to the loss of chemical information from lesioning.

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INTRODUCTION

Interactions between organisms are moderated by a variety of modalities that transmit information from sender to receiver. Signals are the basis of communication between individuals and are defined as any structure that alters the behavior or physiological status of another, the communication itself involving a sender and receiver (Baeckens, 2019). Important information is contained in signals using auditory, olfactory, mechanical, or visual modalities among others. Species have developed complex signaling pathways that are often adapted to the transmission constraints of different environments (Wehner, 1987). Some examples of this are long distance calling among birds in different forests (Mathevon, 2008) and bioluminescent visual signals in deep sea organisms (Ruxton and Bailey, 2005). These signals take a wide variety of forms, but all serve the same function which is conveying information from a sender through the environment to a receiver (Krebs and Davies, 1997).

One of the oldest and most dominant forms of communication is performed with the chemical senses. Chemical signals have a wide range of uses from finding food and mates to navigation, all of which can be determined with chemical scents in the environment (Moore, 2005). Species have developed specialized pathways for chemo-sensation (DeForest, 2012). Insects use primarily chemical signals for kin identification and most social insects conduct communication through pheromones (Richard and Hunt, 2013). Chemical signals contain both specificity and dynamic temporal contrast, meaning that the sender can control the chemical composition of the signal and the timing of dispersal to alter the meaning of the signal to the receiver (Atema, 1995). In addition, chemical signals disperse through the environment through molecular diffusion and bulk flow, as opposed to a wavelength style like audio or visual signals (Atema, 1995). Because of this, chemical signals are highly specialized because they can do what audio and visual signals cannot and are valuable means of communication for many species. However, organisms in certain environments tend to develop similar methods for communication and in aquatic environments there is a bias towards chemical communication.

In the aquatic realm, largely due to a lack of light, chemical communication is a particularly more common modality compared to other senses. Organisms in aquatic environments frequently make use of chemical signals to communicate information efficiently and numerous species use chemosensory as their primary form of communication (Hay, 2009). For example, certain species of fish (*Carassius auratus*) release hormones to self-regulate maturation and to trigger spawning behavior in potential mates (Stacey, 2003). Bloom forming phytoplankton (*Phaeocystis globosa*) use chemical signals to sense when its neighbors are under attack and responds in kind to shift shape until it can no longer be consumed by smaller, predatory ciliates (Long et al., 2007). Additionally, several aquatic organisms lack ears and eyes and as such rely on chemical signals to fill in the gaps and best understand the environment (Hay, 2009). The specificity of chemical signals is exemplified in sea dwelling crustaceans (*Clibanarius vittatus*) which have developed highly specialized peptide signaling systems that can differentiate between a number of similarly shaped chemical molecules and determine the precise pheromone from minute details (Rittchof and Cohen, 2004). Chemical communication is also present in everyday functions, in gastropods chemical signals have been proven to indicate the satiation levels of individuals including specifics of diet and age (Kirsch, 2022). Chemical cues can have effects beyond the individual and can reach into community organization and even ecosystem function as a whole (Hay, 2009). In order to sense chemical signals, many aquatic organisms have developed specialized organs for chemosensory that are adapted to best detect signals in the water column.

Chemosensory information is gained from the environment through the antennules in crustaceans. These appendages contain numerous olfactory sensors known as aesthetascs which are small, hair-like structures located on the lateral antennules that relay chemical sensory information (Mellon, 2012). The ability to detect signals can be affected by several factors including flow speed, rate of flicking, and distribution of aesthetascs along the antennules (Bergman et al., 2006; Mead, 2008; Mellon, 2012). The removal of this source of information has the potential to alter behavioral interactions since damage sustained on the antennules can result in a reduction of olfaction, and overall chemical detection capabilities. Chemical signals influence a wide range of behaviors including aggression, identification, mate choice, and social status (Schneider et al., 2001). Numerous crustaceans including lobsters, crabs, and crayfish utilize urine as an important chemical signal which conveys a range of information about the sender (Simon and Moore, 2007). Previous studies have argued that the ability of contestants to recognize the status of their opponent, which is established via urine release, prior to engaging in combat helps reduce the duration and intensity of aggressive encounters (Goessmann et al., 2000). Urine release in crustaceans is also used in identification and can indicate the social status of combatants (Katoh, 2008). Evidence suggests that crustaceans can control the amount and the timing of urine release, meaning that it is a targeted signal being delivered (Schneider, 1999). Additionally, in agonistic interactions involving crayfish it has been discovered that the blocking of urine release directly from the nephropore leads to increased duration of agonism (Schneider, 2001). With crayfish, urine release from a dominant individual during fights triggers the switch from aggressive to submissive behavior in the receiver, so the urine contains information on the fighting ability of the sender (Breithaupt and Eger, 2002). With the ability to receive information from chemical cues removed, these determining factors would be eliminated from crayfish and would alter their ability to properly assess their opponents and subsequently impact intraspecies interactions. In this study, the goal was to measure the impact of effective chemical communication on regulating the duration and intensity of agonistic interactions in crayfish. For the purpose of this experiment, crayfish were lesioned to remove the ability to detect chemical stimulus and introduced to an opponent. The crayfish's interaction was then observed for duration and intensity of the combat to determine the effects of chemical signaling on agonism.

In instances of conflict, effective signaling is an important determining factor in the level of escalation and duration of the encounter. However, when the ability to receive certain signals is impacted, this can change the approach combatants take to potentially risky confrontations. When crayfish enter into a potential combat situation, the contestants must continually make the decision between remaining in a fight and escalating or exiting the fight and losing it. Ultimately, the main choice which ends the interaction is made by the loser of the fight which must choose to acquiesce and forfeit the fight (Herberholz et al., 2003). These choices are impacted by various intrinsic and extrinsic sources of information available during a contest. For example, contestants' levels of aggression are different depending on whether they are the resident or intruder to the area (Eason and Hannon, 1992), their size compared to their opponents (Wright et al., 2019), the available resources and their quality at stake (Gherardi, 2006; Zhu, 2021), reproductive status (Martin III and Moore, 2010), and several additional factors. Because of the potential complexity of the signals that organisms may incorporate in making the decision to engage in combat, a number of authors have proposed models for the best assessment strategy that animals are employing during fights. A review of work on agonistic behavior across a broad spectrum of animals indicates that there are several strategies or types of assessments that occur for individuals comparing the benefits of winning a fight versus the costs of losing. The intensity of the interaction, temperament, contest duration, and resource holding potential are all important factors when analyzing fighting behavior (Briffa and Elwood, 2009). These traits are especially important when attempting to determine the assessment method best suited for data analysis.

Resource holding potential (RHP) is a metric used to measure the potential for success each combatant has in a contest, and this metric is calculated based on several combined traits including morphology and physiology of each component including both defensive (stamina and endurance) and offensive (strength and body size) attributes (Allen, 2017). All these combined traits add up to the overall potential for success of a winner or defeat of the loser given it's the resources at its disposal at the time of entering a contest. However, RHP is not the only determining factor in the outcome of a contest and there is evidence to suggest that the level of aggression or value of the resource at stake influences the winner (Arnott and Elwood, 2008, Allen, 2017). Some possible indicators of aggressive behavior used to evaluate such behavior in individuals are typically resource holding potential, resource value, and aggressiveness of the individual (Hurd, 2006). The relative importance of each of these traits fluctuates during a given interaction and between interactions. The precise circumstances of the interaction, be it a showdown between males for mates or two individuals competing over nesting ground, are vital to making predictions about the influence of intrinsic or extrinsic forces on individual behavior. These factors can be redefined as the willingness to fight versus the ability or motivation to win (Hurd, 2006). Individuals weigh the anticipated benefits and costs of an interaction prior to engaging in risky behavior that could result in injury or death. Levels of aggression can also be influenced by sex. For example, female crayfish have been shown to be more aggressive when in reproductive form carrying eggs than non-reproductive form females and males in either form

(Figler et al., 1999). Taking all of these factors into consideration is important for determining the proper assessment strategy utilized by individuals entering into potential conflict situations.

The predominant argument for assessment strategies is between self-assessment and mutual assessment. In self-assessment the individual takes stock of its own resources, and it is evidenced that there is a strong positive correlation between an individual's resource holding potential and resource value (Kelly, 2008). Meaning that larger individuals may have access to more resources than smaller ones and as such may have more energy to spare for fighting. Typically, combatants attempt fighting tactics in order from least to most energetically taxing in an effort to conserve resources (Hack, 1997). In shore crabs, larger males defending their territory deterred smaller opponents solely through display whereas similarly sized or larger opponents were engaged in physical aggression for increasingly longer periods depending on the size of the individual (Rovero et al., 2000). The size of the rival has an impact on the length and intensity of the fight to determine a set winner, which ultimately could be tied back to resource holding potential of larger individuals (Arnott and Elwood, 2009). If the contest breaks out into full contact grappling, the ability of the winner to force its opponent to withdraw demonstrates a superior RHP compared to the loser (Briffa and Sneddon, 2006). However, the high cost of injury and loss of energy is often a strong enough deterrent to escalating contests into fighting unless the resources at stake outweigh the costs.

The perceived resource value (RV) of the resources contested over and the resources currently held by each combatant is compared to the relative value of those resources to the self, which is an element of resource holding potential (Briffa and Elwood, 2009). Taking this into consideration, the RV is directly influenced by the RHP, which as stated previously is recognized as a willingness to spend resources. In cases where it is necessary to determine the assessment type, the common process is to compare the RHP of winners/losers to the contest duration as a metric for measuring the assessment type. In both self and mutual assessment, the higher the RHP of the loser, the longer the duration of the contest. This is because the winner is determined by the point at which the loser retreats from the fight and ends the interaction. However, in instances of mutual assessment the winner's RHP is inversely related to the contest duration which is the direct opposite of self- assessment which is still positively correlated to RHP and duration (Arnott and Elwood, 2009).

In mutual assessment situations, the decision of the weaker individual in an interaction to leave the fight is determined in part by their ability to effectively read the RHP of the stronger opponent. By removing the sense of chemical detection in some individuals, this inhibited their ability to make mutual assessments. Chemical signals convey information, in cases where this information is not received it could have an impact on the individual's ability to properly assess its opponents RHP and lead to increased interaction duration. In self-assessment scenarios, each member of the fight is assessing their own RHP and making decisions on whether to act and to what level they can escalate to for a certain cost. When unable to make mutual assessments through chemical information, which is a common moderator for agonistic interactions in crustaceans (Horner, 2008), crayfish may expend more energy to fighting than is metabolically sustainable because they are only aware of their own RHP and must engage the opponent to determine if fighting is worth the effort. In this study, we aimed to investigate the impact of chemical signal reception on assessment strategies in female crayfish during agonistic interactions.

MATERIALS AND METHODS

Animal Collection and Handling

For this experiment, 45 Rusty Crayfish (*Faxonius rusticus*) were collected from the Portage River in Bowling Green, OH, USA (Latitude: 41.3169, Longitude: -83.6083) by seining and hand netting. And 45 Northern Virile Crayfish (*Faxonius virilis)* were obtained from ToledoGoldfish fish farm located in Toledo, OH USA and shipped overnight to Bowling Green State University. Only female Form II (non-reproductive) Rusty Crayfish (1.6 – 2.5 cm) and female Form I (reproductive) Virile Crayfish (2.7- 4.6 cm) were used in this experiment. All crayfish were housed in an environmentally controlled room in the Laboratory for Sensory Ecology. Only crayfish with intact appendages were utilized in this study.

Crayfish were housed in separate plastic containers (25.2×16.2×11.8 cm) in a flow-through recirculating system. Animals were visually and physically, but not chemically, isolated from other crayfish for at least a week to remove any prior social behavior (Schneider et al., 2001). Crayfish were kept on a 12:12 light: dark cycle at ~23°C. All crayfish were fed Manna Pro™ Small World™ Complete guinea pig pellets 3 times a week.

Experimental Design

The purpose of this experiment is to gain a better understanding of the role that chemical cue reception plays in crayfish fight dynamics. To investigate this, crayfish were divided into two groups. The experimental group received an ablation treatment which targeted the chemosensory organs located on the antennules. The control group did not have their chemosensory organs ablated. Combinations of ablated and unablated individuals of both species were placed into fight arenas and their interactions were monitored with a video camera for agonistic behavior. Final pairings for the trial consisted of two different factors: ablated and unablated treatment combined with species producing four groups (Table 2).

Chemosensory Ablation

Treatments were randomly assigned to size matched pairs of animals using a random number generator. Both the control and treatment groups underwent identical handling treatments and the only difference in the procedures was the replacement of salt water (ablations) with dechlorinated, aged tap water (controls). Crayfish were restrained on their dorsal carapace using a clear Plexiglass board with a series of paired holes. The holes had small rubber bands threaded through them which were used to affix the crayfish to the board securely. Once the individual was secured, the lateral and medial filaments of the antennules were placed into a micro-pipette tip (Universal tip, 1000 µl) which was fitted snugly to the rostrum (Figure 1). Crayfish were covered with a damp paper towel to prevent excessive drying and limit stress during treatment.

For the treatment group with ablation, both the lateral and medial filaments of the antennules were placed into the pipette tip which was then filled with 50 ppt saltwater. After a period of 2-hours the saltwater was removed from the pipette tip and replaced with dechlorinated tap water for an additional 10 minutes to complete the lesion. Cells do regenerate after a period of approximately 30 days, to minimize the effects of regrowth all ablated individuals were tested 24-hours post ablation (Kraus-Eppley, 2015). The control group underwent an identical treatment except the salt water was replaced with dechlorinated tap water for both the 2 hour and 10-minute periods. Upon the completion of each treatment, crayfish were returned to their individual housing to recover for 24 hours before being placed in an agonistic assay. Because of the lack of neurological or cellular evidence of this effect, we have termed these behavioral lesions.

Behavioral and Chemical Assay

To confirm crayfish were successfully lesioned behaviorally, a behavioral assay (i.e., flicking of antennules) was performed on all crayfish before fight trials occurred. The behavioral assay consisted of placing a crayfish into a smaller section of a 19-liter aquarium filled with dechlorinated tap water. Tap water used in trials was taken from tanks where it was aged over a period of several days where the chlorine was removed via evaporation resulting in dechlorinated, aged tap water which was safe to use with the crayfish. A camera was placed for a side view of the crayfish and was used to quantify the number of flicks during the assay. To test for the effectiveness of the lesion protocol, crayfish were subjected to two stimuli in consecutive order. The first was 1 ml of dechlorinated tap water delivered to the antennules after a 10 s quiescent period and the second was 1 ml of sardine homogenate also aimed at the antennules at least 10 s post water delivery. The sardine homogenate consisted of 45 grams of sardines (packed in water) homogenized in a blender (Proctor Silex 6 speed blender) with 250 ml of aged tap water.

Fight Trials

Size matched crayfish were paired and placed into a divided tank which served as the fight arena (Figure 2). All crayfish pairs were determined by post-orbital carapace size within 10% for a matched pair of similar sized individuals (Bergman et al., 2003). Size matching animals eliminates the role that total size plays in determining dominance in crayfish (Daws et al., 2002). Previous work has shown that water and odors do not cross between the two sides of a fight tank in the arena (Bergman et al., 2003). Two arenas were placed side-by-side which allowed for a total of four simultaneous trials to be recorded at once. A video camera was mounted approximately 1.3 meters above the arena alongside two dimmable lights to record the fights for subsequent analysis.

The fight arena was constructed of opaque Plexiglas (40 x 40 x 14 cm) which prevented visual contact between individuals prior to introduction. Each arena was divided into four quadrants of equal size (20 x 20 x 14 cm) using opaque retractable walls and was filled with approximately 3.5 gal of aged tap water. Prior to being placed in the arena, one out of every pair of crayfish received an identifying marker on the dorsal side of the carapace with White-Out. All crayfish received small white marks on the flat edge of each chela in order to increase visibility of clasping behavior on camera.

Crayfish were then placed into the arena and were visually and physically isolated for a 15-minute period to allow them to acclimate to the trial conditions (Bergman et al., 2005; Simon and Moore, 2007). Lighting and arena conditions were identical in the acclimation and trial periods. Following acclimation, the appropriate divider was removed, and each pair of crayfish were allowed to interact for 15 minutes. Recording of the interaction began simultaneously with removal of the dividers. These time periods have been shown to produce a dominance relationship in crayfish fights (Bergman and Moore, 2005; Daws et al., 2002). After the trial period completed, the crayfish were moved back into their respective beginning areas, the walls replaced, and crayfish removed from the arena. The fight arena was deconstructed and thoroughly rinsed with hot tap water followed by deionized water to eliminate any traces of chemicals in the water prior to beginning another trial (Bergman, 2003).

DATA ANALYSIS

Analysis of the flicking videos consisted of an observer (blind to the ablation treatment) counting the number of flicks within a 10 s period prior to and after stimulation with the water and fish homogenate. Each fight trial was analyzed by a blind observer to determine the winner and loser of the first bout as well as the duration of the first bout. In addition, the fight dynamics of each first bout was determined using a 13-point ethogram (Table 1). During the analysis, the time to and time at each level of the ethogram was extracted by tracking the transitions between behavioral states on the ethogram. The first bout was defined as the first interaction in which both individuals engaged in chelae to chelae contact for greater than 10 seconds. The conclusion of the interactions occurred when the individuals remained separated by at least two body lengths for at least 20 seconds. Only the first bout was considered in analysis as previous studies have demonstrated that the ultimate winners and losers are established in this time period and subsequent interactions diminish in duration and intensity (Goessman et al., 2000; Huber et al., 2001; Edwards et al., 2003). The loser of the first bout was defined as the individual that retreated the greatest number of times within the defined bout period. All bouts ended with a decisive winner and loser.

Statistical Analysis

All data analysis was done within the programming environment of R (R Core Team 2022). Prior to any statistical analysis three data conditioning steps were performed according to (Zuur et al., 2009). Step one involved the production of Cleveland dot charts to determine if the data contained any outliers. None of the measures showed any outliers. The second step involved the production of histograms, qqplots, and normality tests to determine the normality of the data (Shapiro-Wilk). If the data was not normally distributed, the R function “BestNormalized” was used to select the transformation most likely to produce a normal distribution and after any necessary transformations, the data was rechecked for normality (Peterson, 2021). Flick rates were not normally distributed and were transformed using an orderNorm transformation. For the behavioral measures, not enough fights reach levels 9, 10, and 11 on the ethogram so these were dropped from subsequent analysis. Most of the behavioral variables were not normally distributed. The time to and time at variables along with total contest duration, non-escalated, and escalated durations were transformed using an orderNorm transformation. The final step involved the investigation of any collinearity between the time to and time at variables. None was found, so all the behavioral variables (except for those associated with ethogram levels 9, 10, and 11) were kept within the statistical analysis.

The statistical analysis for the flick rates was performed using a mixed model. Because multiple behavioral measures were collected from a single crayfish, all statistical models were performed using generalized linear mixed models (Zuur et al., 2009). All models run in R used the lmer function from the lme4 package (Bates et al., 2015; R Core Team 2022). The models were constructed with three fixed factors: Stimulus (odor or water), phase (prior to stimulus, post stimulus), and ablation treatment (intact or ablated). Crayfish number was included as the random factor within the mixed model. Following model construction, the outputs were extracted using the anova function from the car package (Fox and Weisburg, 2019).

For the time to and time at fight dynamic analysis, ANOVAs were used where treatment (intact or ablated) and species (*rusticus* or *virilis*) were included in the model. A previous model that included sex as a factor determined that sex had no effect. So, this variable was dropped in the final analysis.

The relationships between participants size (a proxy for their RHP) and total duration and non-escalated durations of bouts were performed using the lm function within R were used to evaluate the assessment strategy hypothesized to be in place across treatment types (e.g., Taylor and Elwood, 2003; Arnott and Elwood, 2009) by comparing our results to expected outcomes seen in Taylor and Elwood, 2003. Initial models were run with either the transformed total duration and non-escalated duration as the dependent variables with full interactions among the various sizes (winner, loser, larger, and smaller size as well as size difference), treatment (intact or ablated), and species (*rusticus* or *virilis*). As with the flicking data, species had no effect on the models, so species was dropped from subsequent analysis. Slopes, adjusted r^2 values, and p values were extracted using the summary command in R.

RESULTS

Flicking

The flick rates of individuals were recorded to measure the response to two stimuli, one mechanical and one chemical, to determine the effectiveness of the treatment. Flick rates of antennules were measured both pre- and post-stimulus using a mixed model analysis. The rate of flicking was a dependent variable while the independent variables were phase (pre and post), stimulus (water vs odor), and treatment (control vs ablation). Animal ID was a random effect. Utilizing an analysis of variance it was determined that there is an interaction between the independent variables (phase, treatment, and stimulus), ($F(_{1,216,0.05}) = 7.69$, $p = 0.006$). A post hoc test was conducted using the emmeans package. Control pre and post odor were found to be different, and post had a higher rate of flicking ($p = 0.0021$). Pre and post odor ablation were not found to be different ($p = 0.43$). Finally, the post odor control flicking was higher than the post odor ablation ($p = 0.034$).

Fight Dynamics

The majority of fights did not escalate beyond levels -2 to 7 (Figure 1) and as such these interactions were classified as non-escalated. Any interaction between individuals that did increase in intensity beyond this point from levels 8-11 was classified as escalated. No fights escalated to the point of levels 10-11. The odds of the initiator also being the winner was calculated using a Tukey HSD multiple proportions test. Fights where the initiator won versus fights where the initiator lost was significant (Chi-Squared = 7.815) so there was a difference between the two. Most often the initiator won the fight (23:9).

Total Duration

Linear regression models reveal that the duration of the fights was significantly increased as both loser size and winner size increased ($F_{(1,34,0.05)} = 6.76$, $p = 0.013$ and ($F_{(1,34,0.05)} = 6.80$, $p = 0.013$). Similarly, duration of fights increased as the larger and smaller size of the combatant increased ($F_{(1,34,0.05)} = 7.2$, $p = 0.011$ and ($F_{(1,34,0.05)} = 6.40$, $p = 0.016$). Interestingly, there was no significant relationship between the size difference of combatants and total duration of the fight ($F_{(1,34,0.05)} = 1.6$, $p = 0.2$). Treatment of the antennules was not significant by itself nor with an interaction of any of the size relationships.

Non-escalated Duration

Unlike the total duration analysis, linear regression models reveal significant interactions between the ablation treatment, the size measurements, and species interactions. The length of the non-escalated duration aspect of a bout was significantly influenced by the interaction between winner size and species ($F_{(1,30,0.05)} = 4.85$, $p = 0.035$) and winner size and treatment ($F_{(1,30,0.05)} = 4.9$, $p = 0.033$). The non-escalated duration was significantly altered by the interaction between all three independent variables (treatment, species, and loser size) ($F_{(1,34,0.05)} = 4.7$, $p = 0.038$). The duration of the fights was significantly increased as both loser size and winner size increased ($F_{(1,34,0.05)} = 6.76$, $p = 0.013$ and ($F_{(1,34,0.05)} = 6.80$, $p = 0.013$). In regard to the size of the loser of the bout, the non-escalated duration was significantly altered by only the interaction between size of the smaller (and larger) combatant and treatment ($F_{(1,30,0.05)} = 4.3$, $p = 0.043$) for smaller combatant; ($F_{(1,30,0.05)} = 4.9$, $p = 0.034$)for the larger combatant). Similar to total duration and size difference of the combatants, the duration of the non-escalated portion of the bout was not influenced by treatment or size, but only species ($F_{(1,30,0.05)} = 21.6$, $p < 0.001$).

Escalated Duration

Escalated Duration

There were no interactions at the escalated level and there were no measurable effects.

DISCUSSION

Our overall findings from this study show that the two most impactful factors on the dynamics of agonistic interactions in crayfish were the ablation treatment and combatant size. These two factors affected the overall duration of the interaction as well as the maximum intensity reached within an encounter. Lesioning of antennules in the treatment groups decreased the fight duration and intensity and demonstrates that chemical cues are an important source of information for assessment strategies in crayfish and the loss of that information alters fight dynamics and assessment strategies.

Overall, the fights did not surpass low intensity in both the control and treatment groups; however, the duration spent interacting at low intensity was longer in the treatment group. These effects apparent with the removal of chemical signal reception indicates that either the exchange of information between pairs is important or some aspect of self-assessment is performed using chemical cues. Assuming that external information on an opponent is important, the exchange of information between combatants controls the escalation speed and intensity during agonistic interactions and is a powerful regulator of fight dynamics (Breithaupt and Atema, 2000). In most decapod crustaceans, chemical signals are assessed through complex sensory pathways which derive from the olfactory sensilla, located on the antennules (Derby et al., 2016). Crustaceans often use chemical signals to communicate a variety of information including social status (Goessmann et al., 2000), reproductive ability (Yen and Lasley, 2010), and threat level (Jurcak and Moore, 2014). With lesioning of the chemosensory organs on the antennules, crayfish are unable to detect chemical signals (Kraus-Epley, 2015). A more detailed analysis indicated that the non-escalated aspects of fights were altered by the absence of chemical information. In particular the overall fight intensity remained lower without chemical cues. Game-theory predicts the relationship between the intensity of fights, the durations of fights, and the type of assessment models used by the combatants.

There are three primary theories used to describe assessment strategies in fights mutual assessment, cumulative assessment, and self-assessment, all of which are based on combatants' ability to win contests, also known as resource holding potential (RHP) (Green, 2018). Each of these strategies establishes the sources of information by which an individual assesses the possibility of winning an encounter either using personal information (self-assessment), information from both self and the opponent (mutual assessment), or the measurement of accumulated costs during an encounter (cumulative assessment). A fight is determined by the point at which one contestant retreats, thus making itself the loser and disengaging from the fight (Briffa and Elwood, 2009). In instances of self-assessment, the loser measures some aspect of RHP solely using private (internal information) and when that personal threshold is met and the animal decides to retreat to minimize further risk. However, in mutual assessment the combatant will decide to retreat after assessing the difference between its own RHP and its opponent's RHP (Taylor and Elwood, 2003). The exchange of information between opponents carries the cost of revealing information as well as securing information to and from the opponent. The lesioning treatment removed crayfish's ability to receive chemical signals via the antennules. If chemical signals (such as urine) are important sources of RHP information, then both self and mutual assessment would be disrupted which was seen in these results. From here the question becomes whether the crayfish were utilizing self or mutual assessment with the chemical signals.

In our study, there was a significant effect of the treatment on the duration of non-escalation intensity fights. This treatment effect was seen in conjunction with winner size and species. These results could be explained if crayfish use self-recognition via their own chemical signals and utilize that information in a self-assessment strategy. Chemical self-recognition has been observed in cichlids (*Pelvicachromis taeniatus*) which demonstrated a preference for their own scent over known scents of siblings or unknown strangers, which indicates a level of self-cognizance (Thünken et al., 2009). The chemical components in crustacean urine include a wealth of metabolites which are indicators of the physiological state of the sender (Breithaupt and Atema, 2000). The sender could be investigating its own odor output and using that as a gauge for its stamina levels during fights. Both before and at intervals during a fight several chemical releases have been quantified and include glucose, glycogen, and lactic acid levels (Prenter et al., 2006; Briffa and Elwood, 2005). Given the chemical composition of urine, these signals could be utilized as deliberate signals of physical health to broadcast an individual's strength prior to and during fights. If so, these signals would moderate the duration of the fight as each contender sends and receives those signals. This, however, would be employed in a mutual assessment strategy, which we did not see evidenced by the data. Thus, at this point, these results do not allow the distinction between self and mutual assessment. Beyond chemical signals, these animals may be using other sources of information.

Visual and tactile cues are utilized in addition to chemical cues during agonistic interactions. These cues take many forms including offensive posturing to defensive postures, antennae drumming, and even fleeing, all of which communicates information in agonistic interactions (Bruski, 1987). While the informational content of these cues or signals is currently unknown, the removal of this information alters fights. For example, freshly molted crustaceans (*Gonodactylus bredini*) demonstrate increased meral spread behaviors in the presence of potential threats, despite having a soft body. The interpretation is that the meral spread is bluffing the preparedness of the crayfish to fight in an attempt to discourage attacks while vulnerable (Steger and Caldwell, 1983). So, the importance of visual cues in deterring opponents from entering combat is important in the life cycle of crayfish. The antennae on crayfish have multiple uses, but during fights male crayfish (*Orconectes virilis*) have been observed engaging in antennal waving as a submissive display which resulted in its release from grappling positions by its opponent (Bruski and Dunham, 1990). This waving and drumming is both a visual and tactile display to minimize injury and end the fight. There are a variety of dominant and submissive signals that crustaceans release during fights and interactions are multimodal, but the importance of each signal varies depending on the context of the fight. In crayfish (*Orconectes rusticus*) with impaired sensory modalities including vision, touch, and olfaction, animals were able to form dominance hierarchies successfully with olfaction removed while the other two modalities remained intact. However, when reduced to only one physical modality (vision or touch) with chemical sensory removed the crayfish were unable to establish dominance successfully (Callaghan et al., 2012). These results indicate that chemical cues are important in regulating social hierarchies, but also that visual and tactile cues work in conjunction with chemical cues to convey messages about dominance successfully.

The size effect on increased fight duration seen in our study may be due to relative RHP of larger individuals. Increased size is related to larger RHP and the ability to fight longer. In our study, the only resource available to the crayfish was territory within the fight arena which is likely not important. Some research has shown that crayfish with a greater RHP are more likely to initiate and win fights motivated by territory disputes; however, in the case of this study there was no territory to fight over (Briffa and Elwood, 2009). The effect of size could be due to the larger cost of fight escalation for larger individuals with similarly sized opponents which could deal a significant amount of damage if the assessment strategy utilized by the initiator is inaccurate (Arnott and Elwood, 2009). Fighting is a costly endeavor and generally animals make every attempt to prevent escalation and instead use threat displays to resolve conflict (Gardner and Morris, 1989). Additionally, inaccurate self-assessment of size can play a role in fight dynamics as observed in rusty crayfish (*Orconectes rusticus*) which when placed in small shelters relative to body size prior to engaging in fights were more likely to overestimate their body size which led to an increased percentage of fights won (Percival and Moore, 2009). The crayfish perceived themselves as bigger than their opponents and acted accordingly with increased aggression, having valued their ability to win (RHP) as higher than their opponents even though that was untrue. The energetic cost of fighting can lead to lasting effects on the individual and its ability to win (RHP) is influenced by the quality of resources contested over (Riechert, 1988). However, the physical differences in size among crayfish did not have as significant of an effect on the fight dynamics as the removal of chemical sense.

Olfactory cues are important signals in aquatic environments and are utilized in a wide range of behaviors. For example, crayfish are capable of individual recognition of familiar individuals and the social status of opponents through odor (Schneider et al., 2001). The past dietary history of a predator can be discerned through chemical cues by prey and subsequently alters their behavior (Kamio et al., 2022). Incidentally, chemical signals are considered more honest signals than other modalities because of the direct tie to the physiological state of the sender. However, the release of this physiological information by chemical signals can be controlled through the periodic release of chemicals during interactions (Breithaupt and Atema, 2000). The control and more honest nature of chemical signals can make them important sources of information for aquatic organisms. The importance of olfaction in crayfish is highlighted by the highly specialized olfactory pathway through which crayfish capture odor molecules in the water and incorporate them into the olfactory neurons (Schmidt et al., 2008).

Conclusion

In summary, there can be a variety of signals that crayfish utilize when engaging in agonistic behaviors. However, when chemical signals are removed as a form of sensory reception, this ablation has a measurable effect on fight duration and intensity. The integration of information from a number of sensory modalities is important, however; chemical cues in crayfish appear to influence a wide range of social behaviors in a unique fashion that mechanical and visual systems do not. While the results presented here make it difficult to definitively say that self-assessment was being utilized by the crayfish during interactions, the results strongly support that chemical signals are vital to mediating fights and their outcomes. Crayfish may be measuring their own fighting ability through the information contained in their urine, and without that information, may be less willing to engage in intense fights and spend more time engaging in low intensity behavior which preserves energy and minimizes risk. Larger crayfish have more energy resources than smaller crayfish which may explain why their duration was longer than smaller crayfish. Additionally, the species differences observed may be due more to size as opposed to form since on average the virile crayfish carapace's sizes were larger than the rusty crayfish and size was observed to be significant. However, the overall effect of chemical signals in fight moderation were observed to be linked to increasing the duration and reducing the intensity of agonistic interactions between females.

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APPENDIX A. FIGURES

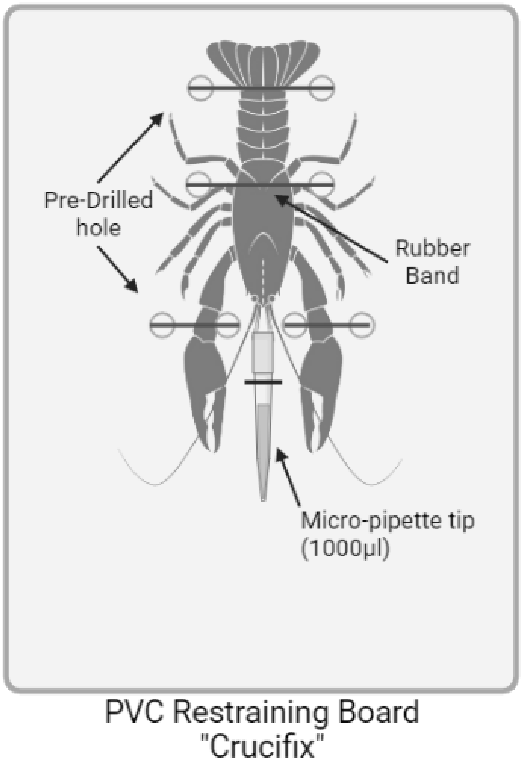

Figure 1. Schematic showing the restraining board used to secure the crayfish while receiving the lesion treatment. Crayfish were placed on their dorsal surface, stomach up, and secured to the board using rubber bands that were fed through pre-drilled holes in the board. Rubber bands secured the crayfish at each chelae, thorax, and the telson. A micro-pipette tip (1000 µl) attached to the board via rubber band was fitted to the end of the rostrum. The antennules were inserted into the pipette tip which was then filled with the appropriate solution. A damp paper towel was placed over the board and crayfish to prevent excessive drying during the treatment period.

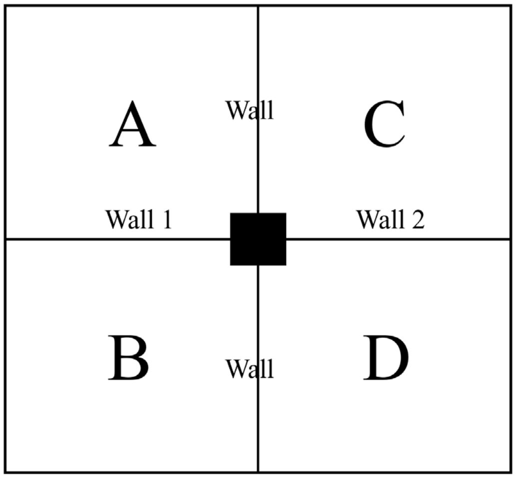

Figure 2. Schematic of the fight arena used for all agonistic encounters. The arena consisted of four individual sections with removable divider walls. Previous work has shown that there is no dispersion of water between the four areas before the walls are removed, thus crayfish were physically and chemically isolated by the dividers for the duration of the acclimation. Only two walls (e.g., 1 and 2 in diagram) were removed to allow individuals to engage. Fight arenas were monitored by an overhead camera (1.3 m above) which recorded the fight for the 15-minute duration.

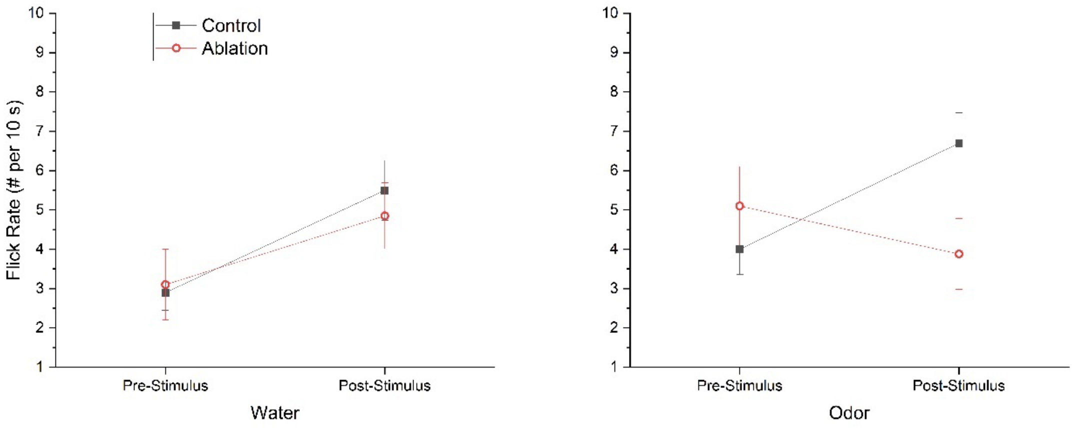

Figure 3. Mean (± SEM) number of flicks prior to (left hand pair of symbols) and post (right hand pair of symbols) stimulation with water (left hand graph) or odor (right hand graph). Solid red circles represent crayfish that had sham lesions and solid black squares represent crayfish with antennules ablated. The ablation treatment significantly reduced the response to odor stimuli, but not water stimuli (Three-way ANOVA: $F_{(1,216,0.05)} = 7.69$). Tukey-HSD post hoc analysis showed that the flick rate for control crayfish pre and post odor were found to be significantly different and that crayfish in the ablation treatment did not response to odor stimuli ($p = 0.43$). Finally, the number of flicks for crayfish in the sham (control) treatment was higher during odor stimulation than crayfish that received the ablation treatment ($p = 0.34$).

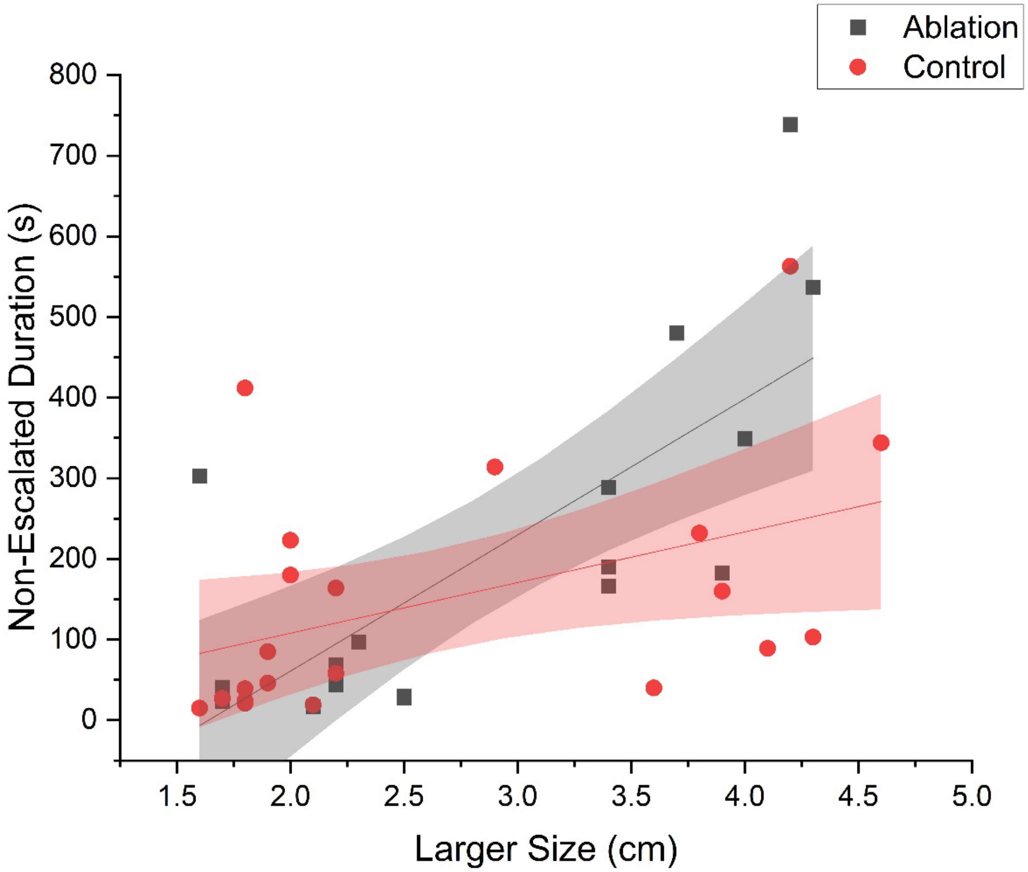

Figure 4. Linear regression of the non-escalated duration of crayfish fights as a function of the larger size. The solid red circles represent crayfish that had sham lesions (control) and solid black squares represent crayfish with antennules ablated (treatment). Colored shading represents the 95% confidence interval for each treatment type (Red = control, Black = ablation). Non-escalated intensity is the total duration of the fight where combatants were at lower intensities on the ethogram (-2 to 7: Table 1). A linear regression was run which yielded the following results, for the ablation, adjusted r squared = 0.52, $p < 0.0001$, and control, adjusted r squared = 0.15, p = 0.045. A mixed model revealed that there was a significant interaction between size, treatment, and duration for larger combatants ($F_{(1,30,0.05)} = 4.9$, $p = 0.034$).

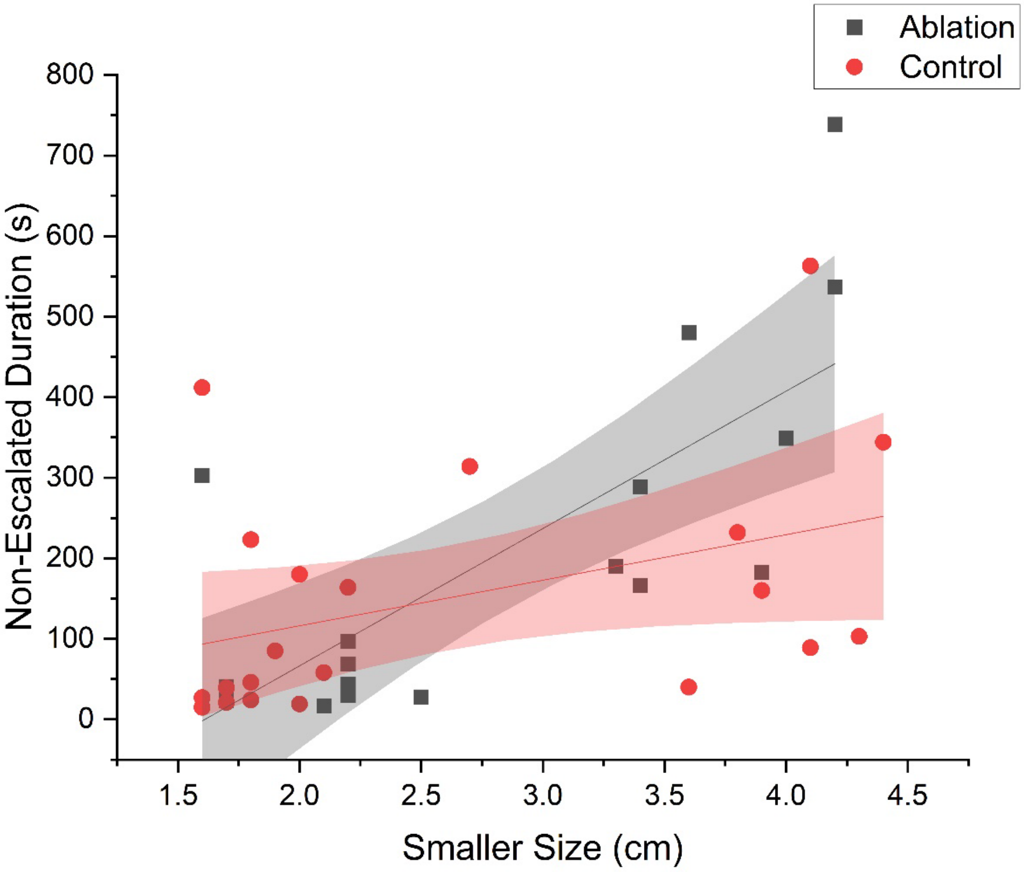

Figure 5. Linear regression of the non-escalated duration of crayfish fights as a function of the smaller size. The solid red circles represent crayfish that had sham lesions (control) and solid black squares represent crayfish with antennules ablated (treatment). Colored shading represents the 95% confidence interval for each treatment type (Red = control, Black = ablation). Non-escalated intensity is the total duration of the fight where combatants were at lower intensities on the ethogram (-2 to 7: Table 1). A linear regression was run which yielded the following results, for the ablation, adjusted r squared = 0.54, $p < 0.0001$, and control, adjusted r squared = 0.12, $p = 0.07$. A mixed model was run which revealed that there was a significant interaction between size, treatment, and duration for smaller combatants ($F_{(1,30,0.05)} = 4.3$, $p = 0.043$).

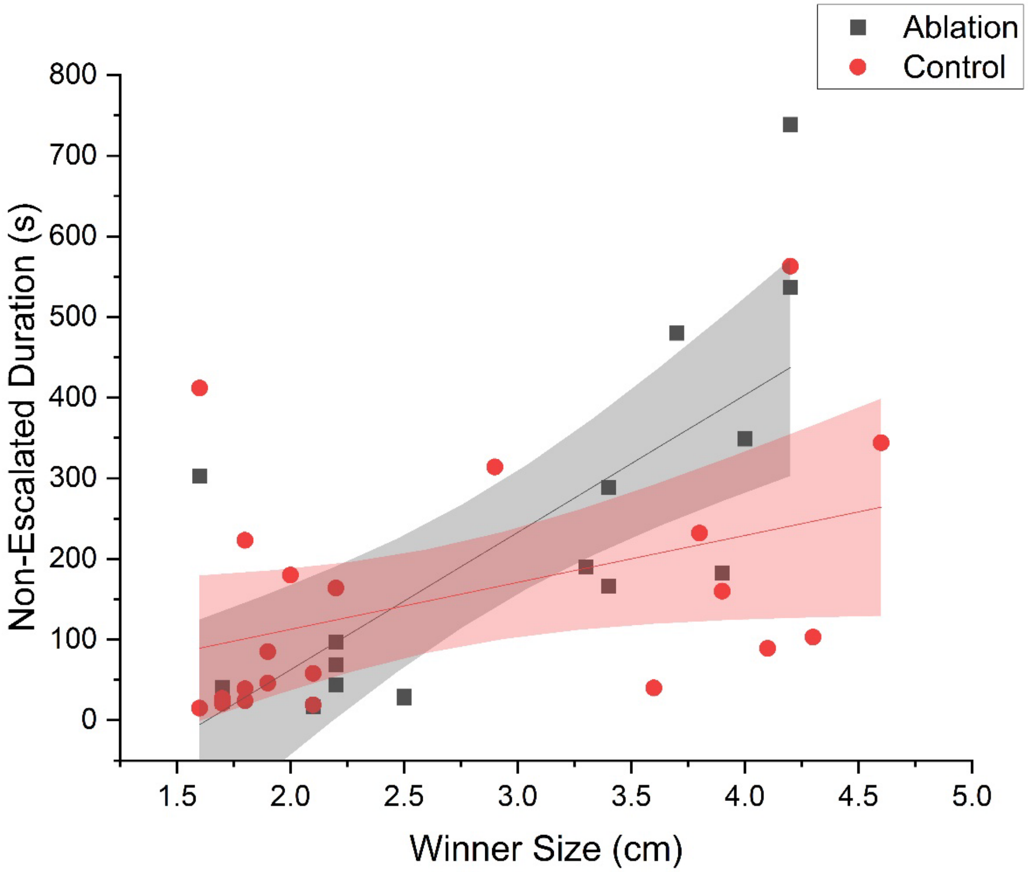

Figure 6. Linear regression of the non-escalated duration of crayfish fights as a function of the winner size. The solid red circles represent crayfish that had sham lesions (control) and solid black squares represent crayfish with antennules ablated (treatment). Colored shading represents the 95% confidence interval for each treatment type (Red = control, Black = ablation). Non-escalated intensity is the total duration of the fight where combatants were at lower intensities on the ethogram (-2 to 7: Table 1). A linear regression was run which yielded the following results, for the ablation, adjusted r squared = 0.53, $p < 0.0001$, and control, adjusted r squared = 0.13, $p = 0.06$. A mixed model revealed that the duration of non-escalated fights was significantly increased by winner size ($F_{(1,34,0.05)} = 6.80$, $p = 0.013$). Additionally, the interaction between winner size and species ($F_{(1,30,0.05)} = 4.85$, $p = 0.035$) and winner size and treatment ($F_{(1,30,0.05)} = 4.9$, $p = 0.033$).

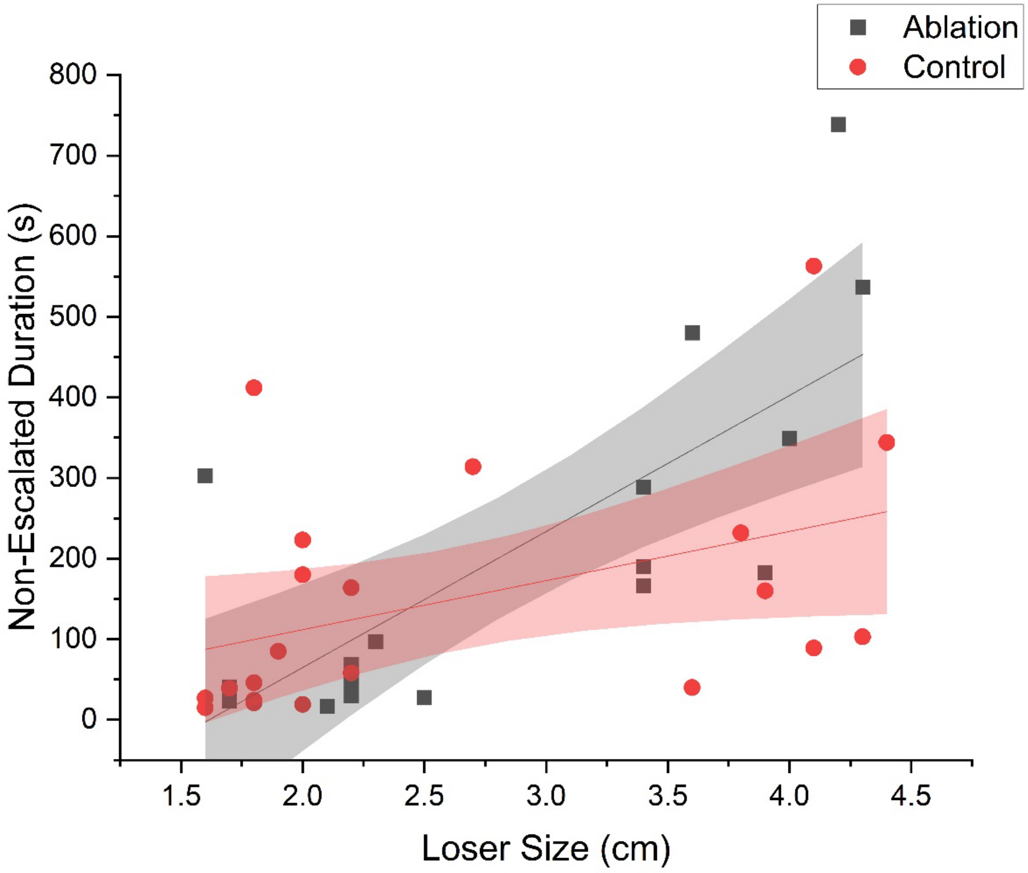

Figure 7. Linear regression of the non-escalated duration of crayfish fights as a function of the loser size. The solid red circles represent crayfish that had sham lesions (control) and solid black squares represent crayfish with antennules ablated (treatment). Colored shading represents the 95% confidence interval for each treatment type (Red = control, Black = ablation). Non-escalated intensity is the total duration of the fight where combatants were at lower intensities on the ethogram (-2 to 7: Table 1). A linear regression was run which yielded the following results, for the ablation, adjusted r squared = 0.53, $p < 0.0001$, and control, adjusted r squared = 0.14, $p = 0.05$. A mixed model revealed that the duration of non-escalated fights was significantly increased as loser size increased ($F_{(1,34,0.05)} = 6.76$, $p = 0.013$). However, the duration was also

significantly increased by the interaction between species, treatment, and loser size ($F_{(1,34,0.05)}$ = 4.7, p = 0.038).

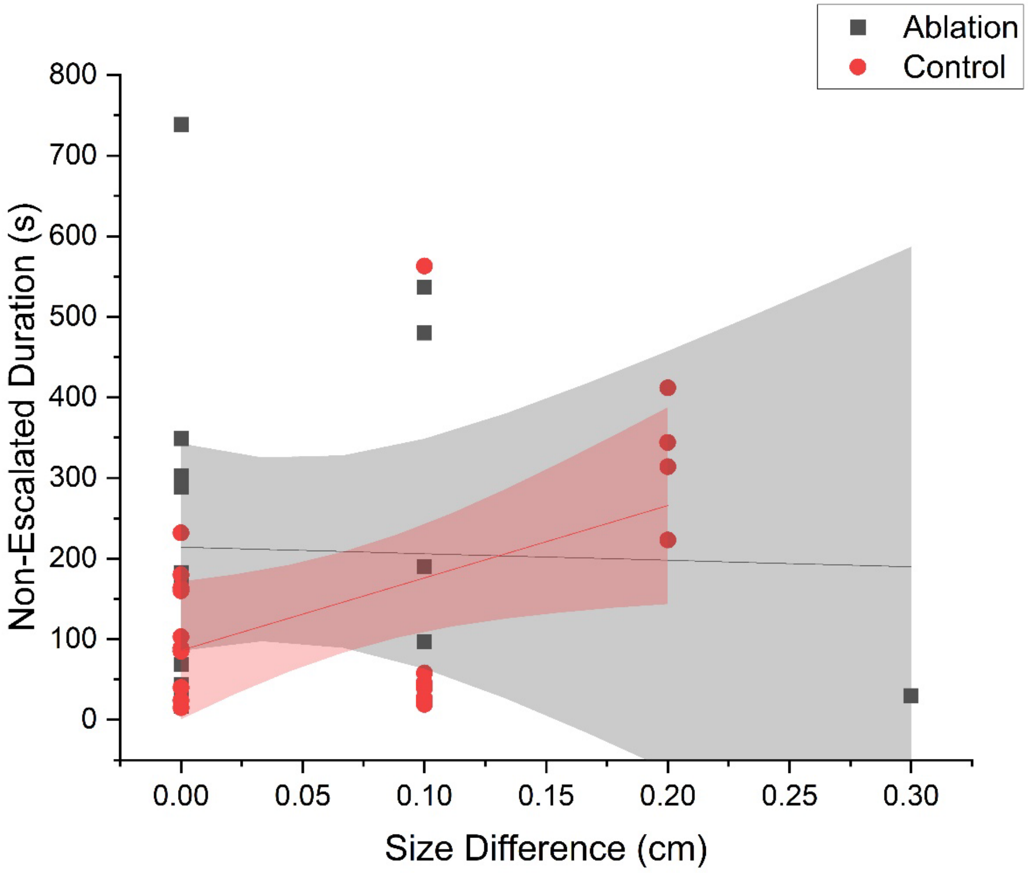

Figure 8. Linear regression of the non-escalated duration of crayfish fights as a function of the size difference between combatants. The solid red circles represent crayfish that had sham lesions (control) and solid black squares represent crayfish with antennules ablated (treatment). Colored shading represents the 95% confidence interval for each treatment type (Red = control, Black = ablation). Non-escalated intensity is the total duration of the fight where combatants were at lower intensities on the ethogram (-2 to 7: Table 1). The size difference was measured by the difference in post-orbital carapace lengths between the winner and loser of the fight. A linear regression was run which yielded the following results, for ablation, adjusted r squared = 0.07, p

= 0.91, and control, adjusted r squared = 0.18, p = 0.03. When considering the size difference, a mixed model revealed that the duration of the non-escalated portion of the bout was not influenced by treatment or size, but only species ($F_{(1,30,0.05)} = 21.6$, $p < 0.001$).

APPENDIX B. TABLES

13 Level Ethogram

Behavior name	Intensity	Behavior Description
Tailflip	-2	Rapid movement of the tail in reverse without chelae grab
Slow Retreat	-1	Lower intensity walking away from opponent
Slow Approach	1	Lower intensity walking toward opponent
Rapid Approach	2	Quick high intensity approach with meral spread
Closed Chelae Touch	3	No forceful movement, just chelae touches
Antennal Whipping	4	Hitting the opponent with Antennae
Stiff Arm	5	Close chelae straight forward holding opponent away
Closed Claw Boxing	6	Pushing with chelae that are closed
Open Claw Touching	7	Open chelae touching; no pushing; no closing
Open Claw Boxing	8	Open chelae pushing, no closing
Grabbing at Appendages	9	Chelae closed around appendage, no pulling
Tearing of Appendages	10	High intensity and rapid movement attempting to rip claws or appendages
Inversion	11	Flipping opponent over

Table 1. Ethogram used to analyze fight dynamics of crayfish is modified from Bergman et al., 2003. Intensity levels -2 and -1 represent behaviors exhibiting the ending of an agonistic interaction. Levels 1-7 represent intensity levels considered to be non-escalated levels, whereas levels 8-11 are considered escalated levels of intensity

Treatment Groups

Species	**Treatment**	**Trials (matched pairs)**
Faxonius virilis	Ablated	N = 8
Faxonius virilis	Unablated	N = 8
Faxonius rusticus	Ablated	N = 8
Faxonius rusticus	Unablated	N = 8

Table 2. Ns for the different treatments outlined in the methods. All pairs were matched within 10% of their carapace size.