The Effects of Atrazine on Agonistic Behavior in Crayfish (*Orconectes virilis*) in a Dynamic System

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Atrazine is the most commonly found herbicide in groundwater in the United States. Its adverse effect on aquatic organisms is well-researched. This experiment looks at how atrazine effects the agonistic behavior of Orconectes virilis in a dynamic system. We expected the atrazine-exposed crayfish to be less aggressive and therefore less successful in agonistic encounters with naïve crayfish. Crayfish of similar size were paired together to engage in a recorded fight, one having been exposed to 200 ug/L atrazine for 24-hours and one just to standard stream conditions. We found no statistical evidence to support our hypothesis that atrazine lowers the aggressive behavior in O. virilis. Regardless of our results, there is enough supporting evidence to show how atrazine negatively affects not only crayfish, but all levels of aquatic ecosystems, and it should not be used as heavily as an herbicide in the United States..

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Atrazine is the most commonly found herbicide in groundwater in the United States. Its adverse effect on aquatic organisms is well-researched. This experiment looks at how atrazine effects the agonistic behavior of *Orconectes virilis* in a dynamic system. We expected the atrazine-exposed crayfish to be less aggressive and therefore less successful in agonistic encounters with naïve crayfish. Crayfish of similar size were paired together to engage in a recorded fight, one having been exposed to 200 ug/L atrazine for 24-hours and one just to standard stream conditions. We found no statistical evidence to support our hypothesis that atrazine lowers the aggressive behavior in *O. virilis*. Regardless of our results, there is enough supporting evidence to show how atrazine negatively affects not only crayfish, but all levels of aquatic ecosystems, and it should not be used as heavily as an herbicide in the United States.

Introduction

Atrazine is a synthetic herbicide used to kill broadleaf weeds in an agricultural setting (USEPA, 2007). It works by being taken up through the roots of the plants and halting photosynthesis once it enters the shoot and leaves (ATSDR, 2003). Atrazine is the most commonly used herbicide in North America, mostly in the Midwestern Corn Belt with more than 25 kilograms of atrazine used per square kilometer (USGS, 1998). Once applied to crops, rainfall and irrigation can cause agricultural agents to enter aquatic ecosystems through runoff. As a result, atrazine is the most prevalent herbicide found in both streams and groundwater in the

United States (Gilliom et al, 2006). Atrazine also has a very high aquatic half-life, taking it years to completely disappear from groundwater (Comber, 1999). Reports from the EPA and the Agency for Toxic Substances and Disease Registry (ATSDR) have suggested that atrazine cannot be shown to cause cancer in humans but can cause disruptions in both the endocrine and reproductive systems (EPA, 2006; ATSDR, 2003).

Numerous studies have showed how atrazine runoff into streams and standing water has negative effects on the wide variety of organisms inhabiting aquatic ecosystems (Christopher and Bird, 1992) (Dewey, 1986; Detenbeck et al, 1996; Graymore et al, 200). As a photosynthesis inhibitor, atrazine has been found to impede growth and eventually lead to the death of multiple species of macrophytes (Correll and Wu, 1982) and freshwater algae (Tang et al, 1997). Removing primary producers from aquatic habitats has detrimental consequences on organisms higher up on the food chain. Atrazine also directly affects those larger aquatic organisms. A controversial study found that exposure to atrazine results in hermaphroditism in tadpoles, which became widespread news as the public questioned the potential impact atrazine could have on humans (Hayes et al, 2010).

Crayfish have been identified as a keystone species in certain aquatic habitats (Nisikawa, 2010). Therefore, any introduced toxin that negatively affects crayfish can be detrimental to the entire ecosystem. The effect of atrazine on *Orconectes rusticus*, an invasive freshwater crayfish species found in the midwestern United States and Canada, has been thoroughly documented, specifically its effect on foraging behavior (Belanger et al, 2015) and conspecific odor recognition (Belanger et al, 2017). Atrazine has also been found to retard growth in juvenile crayfish, as well as cause sexual differentiation favoring females over males, similar to the findings in Hayes’ 2010 study on tadpoles (Mac Loughlin, 2016). Measuring the effect of atrazine on a native, non-invasive species of crayfish, such as *Orconectes virilis*, is less prevalent due to the rusty crayfish becoming more abundant in the Midwest.

Our research focused on the effects of atrazine on the agonistic behavior of *O. virilis* in a dynamic stream system. We hypothesized that exposure to atrazine at high concentrations will negatively affect the aggressive behavior of the virile crayfish, rendering them more docile and less-able to defend themselves. We expected to observe this effect due to the negative impact atrazine has on the chemosensory response of crayfish (Belanger, 2016), and how crayfish rely heavily on chemical sensing during agonistic encounters (Zulandt et al, 2001).We tested our hypothesis by exposing crayfish to a solution of atrazine for 24 hours and having them fight crayfish that had been not been exposed.

Materials/Methods

Female *Orconectes virilis* were collected from Maple Bay State Forest Campground, Cheboygan, MI, a boat launch site on the western end of Burt Lake, at 23:00. The carapace and right chelae of each crayfish were measured before they were separated in the holding tank. The crayfish were separated to avoid them coming into contact with one another before the recorded bouts.

An artificial stream was set up at the UMBS Stream Lab to mimic the conditions of a dynamic system (Figure 1). The stream had two separate chambers (120 x 39.5 x 23 cm), each exposed to similar flow rates of water from the Maple River. The left stream, used for naïve tests, flowed at 4.70 liters per minute. The right stream, exposed to atrazine, was 4.32 liters per minute. Each chamber was separated by a plexiglass divider to allow for two crayfish to be stored in each chamber at once and for two tests to be run every 24-hours. Both sides were left unaltered to run the two control trials, and the final two trials had both chambers used for atrazine exposure. For the atrazine exposure chamber, 10 liters of 0.2% atrazine mixture was created by diluting 0.5 liters of 4% atrazine with 9.5 liters of water. The solution was stored in a three-gallon bucket that sat on the right chamber wall. A small tube delivered the atrazine solution to the bottom of the head of the stream at a rate of 4.32 milliliters per second. The atrazine was delivered during the exposure period until all ten liters of solution was introduced to the artificial stream, which would take approximately 38 minutes.

A total of eight trials were run. The control group was two trials of a naïve vs naïve crayfish. There were four experimental trials, in which a naïve crayfish fought a crayfish that had been exposed to atrazine for 24 hours. Finally, two trials of an exposed crayfish fighting with a second exposed crayfish were recorded. Crayfish with similar carapace and chelae lengths were paired for tests. For the experimental trials, two crayfish would be tethered in the naïve stream, and two tethered in the exposed stream. The crayfish would remain in the artificial stream for a 24-hour exposure period. Once 24-hours had passed, the paired crayfish were collected and placed into an arena (40.5 x 20.5 x 15 cm) containing three liters of river water, ensuring both crayfish were completely submerged (Figure 2). Once both crayfish were submerged in the container, a door was raised, allowing them to interact. Each bout lasted five minutes before the crayfish were returned to their respective containers and placed back into the holding tank. Each bout was filmed to be observed and scored. For the final four trials, a 3-gallon bucket filled with 3 liters of water was used instead of the arena due to the arena no longer being available.

The fights were scored by assigning point values to specific aggressive and passive actions committed by the crayfish after Bergman and Moore, 2003. Once all of the trials had been scored, the mean score was calculated for both the naïve and exposed crayfish. We ran a Wilcox test to determine if there was a significant difference between the mean agonistic behavior of the naïve and atrazine exposed crayfish. We also ran a paired T-test to look at the difference in fight values between each individual experimental trial (naïve vs atrazine). All data were analyzed in R Studio, version 3.3.2.

Figure 1. The artificial stream set-up at the UMBS Stream Lab

Figure 2. The arena used to host the crayfish bouts

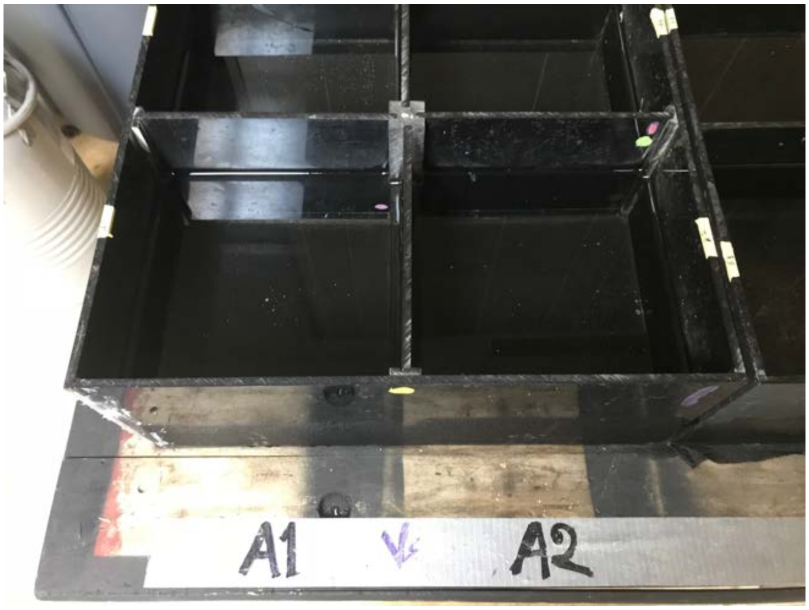


Figure 3. Point values used when scoring the crayfish bouts

Intensity level	Description
−2	Tailflip away from opponent or fast retreat
−1	Slowly back away from opponent
0	Ignore opponent with no response or threat display
1	Approach without a threat display
2	Approach with threat display using meral spread and/or antennal whip
3	Initial claw use by boxing, pushing, or touching with closed claws
4	Active claw use by grabbing opponent with open claws
5	Unrestrained fighting by grasping and pulling opponent's claws or appendages

Results

We observed no significant difference between the mean fight scores of naïve *O. virilis* and those exposed to atrazine (W = 61.5, p-value = 0.9736; Figure 4). The mean fight score for the naïve crayfish was 6.36, and that for the exposed crayfish was 6.72. There was also no significant difference between the fight values of each individual experimental trial (T = 1.15, df = 6, p = 0.292; Figure 5).

Figure 4. Box plots comparing the mean fight scores of naïve (control) and exposed crayfish

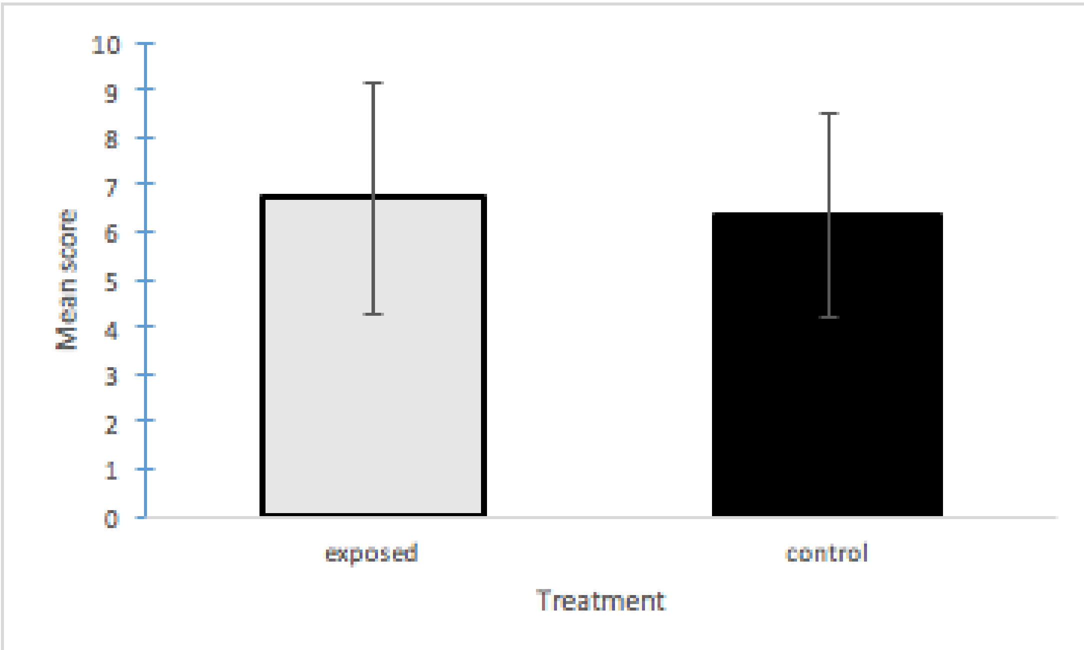
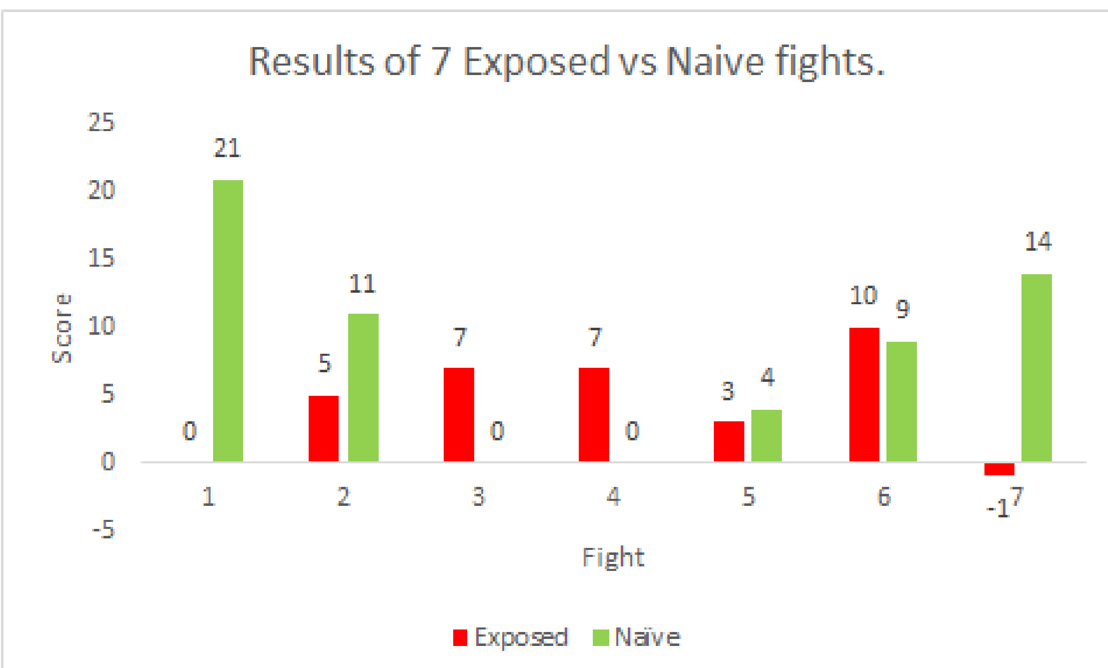


Figure 5. Results of the seven naïve vs. exposed crayfish fights



Discussion

Research has shown that atrazine has a negative effect on the chemosensory abilities of crayfish in both short and long-term exposure experiments (Belanger, 2015) (Belanger, 2016). Crayfish rely heavily on chemical signals for finding mates (Belanger and Moore, 2009), avoiding predators (Keller and Moore, 1999), and determining social status through agonistic interactions (Zulandt et al, 2001). Another herbicide, MetaChlor, has also been shown to negatively affect crayfish during agonistic encounters (Cook and Moore, 2008). From these previous studies we developed the hypothesis that acute atrazine exposure would lessen the agonistic behavior in *Orconectes virilis*, rendering them less-able to defend themselves against naïve crayfish.

Our results did not show a statistically significant relationship between the effect of short-term atrazine exposure in the agonistic behavior of female *O. virilis*. It is worth noting that both studies used male *Orconectes rusticus* instead of the female *O. virilis* that were used in this experiment. *O. rusticus* is found to be more aggressive than other species of crayfish, which helps them be such a prolific invasive species (Bergman and Moore, 2003). As a sexually dimorphic species, male crayfish are also more likely to fight other males for both resources and females, and therefore most studies are focused on male agonistic interactions (Draud et al, 2004). It is possible that female aggressive behaviors are just as strong in comparison to the males, but further research is needed on female crayfish agonism.

Previous studies on the effects atrazine exposure on crayfish used longer periods of atrazine exposure (Belanger et al, 2015; Belanger et al, 2016; Belanger et al, 2017). These three studies on how the crayfish chemosensory system responds to atrazine performed by Rachelle Belanger had much longer exposure times compared to our experiment (2015, 2016, 2017).

Belanger's experiments exposed crayfish to atrazine for 96 and 72-hour periods, while we exposed for 24 hours. Also, we only exposed the crayfish to 10 liters of atrazine at a flow rate of 4.32 milliliters per second, therefore the atrazine would be depleted after 38 minutes. This further reduces the exposure time and could explain our results of the atrazine not having an effect on the crayfish.

The three Belanger experiments (2015, 2016, 2017) also used static systems when exposing the crayfish to atrazine, injecting atrazine solution into the small containers that the crayfish were stored in to ensure that each crayfish got an equal concentration of exposure. Our experiment utilized a dynamic system for the exposure periods to better mimic the environmental conditions of atrazine being introduced to a stream by agricultural runoff. Although atrazine can remain in the substrates even after it has flowed out of the stream (Mengjie et al, 2017), using a dynamic system doesn't ensure that each crayfish will be equally exposed, or that the crayfish will be exposed during the entire period. This is another potential reason for our data being inconclusive.

There is a plethora of research showing the adverse effects that atrazine has on the various species in aquatic ecosystems, and it's still the most common pesticide in groundwater in the United States (Gilliom et al, 2006). The EPA is ignoring the potential hazards it poses to people by discrediting research that opposes atrazine usage on crops (Wu et al, 2009). Although our research on the adverse effects of atrazine on crayfish did not yield significant results, there is enough evidence in supporting research to deem our experiment as an outlier. This substance has already been banned in the European Union due to its potential toxicity (Bethsass and Colangelo, 2006), and continuing research like ours will hopefully lead the EPA and the United States to follow in their footsteps for the betterment of human health.

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