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The Effects of Triclosan on Crayfish (Orconectes propinquus)

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*Senior Honors Thesis**

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Introduction

Every day, thousands of different chemicals are used in the production of consumer goods. Nearly 80,000 of these chemicals have either never been tested or have not been tested sufficiently by the FDA for toxicity in humans (Zarlin, 2012). One of these chemicals is 5-chloro-2-(2,4-dichlorophenoxy)-phenol, otherwise knows as Triclosan (TCS, Figure 1). In recent years, the use of TCS in products such as soaps, shampoos, and other personal care items has dramatically increased due to its antibacterial properties (Cherednichenkoa et al., 2012). Therefore, the majority of its disposal is directly in wastewater and sewer systems, eventually being sent to wastewater treatment plants (Orvos et al., 2002). However, TCS is rarely removed from wastewater by the treatment plants, and levels of the chemical are now being detected in aquatic ecosystems at levels as high as 0.500 mg/L (Samiksha et al., 2010; Dann et al., 2011; Foran et al., 2000).

Not only is TCS being detected in U.S. waterways and within various aquatic organisms, it is now also being detected in many humans. Although not much research has been done on its toxicity to humans, TCS has been demonstrated to be very toxic to different aquatic species such as fish, water insects, and algae (Adolfsson-Erici, 2002). In addition, the compound can also be degraded in the aquatic environment by sunlight into other toxic chemicals including dioxins, chloroform, and di and tri-chlorophenols, which have been flagged as priority pollutants by the EPA (Dann et al., 2011).

Along with TCS being a toxin to many small aquatic organisms that play a crucial role in food webs, humans should also be concerned about TCS as one of the main effects of TCS

in aquatic organisms has been endocrine disruption (Samiksha et al., 2010). This raises concern over the possibility of similar effects when bioaccumulating into humans. Due to a number of recent alarming discoveries, the safety of TCS is currently being re-evaluated (EPA, 2012). We believe the chances are high that TCS will also affect crayfish and have planned accordingly for future studies to better understand this effect.

Many aquatic organisms have been examined for effects that TCS may have on them, but one critical aquatic animal that has thus far been ignored is the crayfish. Crayfish are an important organism in many freshwater ecosystems. They have a significant role in the food webs as both predator and prey and thusly have major impacts on their habitat (Usio & Townsend, 2002). We hypothesize that TCS will have toxic effects on crayfish, *Orconectes propinquus,* similar to other previously studied aquatic organisms. Therefore we have examined the effects that the chemical TCS has on the crayfish species *Orconectes propinquus,* which is common in the state of Michigan. If TCS does indeed have toxic effects on a key species of aquatic ecosystem's, then the use of this antibacterial ingredient that offers little to no benefit over plain soaps (Kuehn, 2010), will be questioned even further.

Another focus of our study was to determine the effects that TCS on mate attraction and possible indirect effects on the endocrine system of crayfish. Specifically, we tested the attraction towards female and male odors by male crayfish after exposure to TCS. Crayfish rely heavily on olfaction for finding mates for reproduction (Fingerman, 1995), so disruption of olfaction or the physiology of their endocrine systems could be detrimental to

the ultimate survival of the species. Specifically, crayfish heavily rely upon their sense of smell for orientation in finding food and mates (Grasso, 2002). The ability of the crayfish to locate food and mates after exposure to TCS was examined to determine if there were effects on their olfactory system. Based on previous studies of other toxins in our lab, we hypothesize that TCS will disrupt male crayfishes' attraction towards females and also disrupt their ability to locate food. Lastly, a lethal concentration where 50% of exposed crayfish died (LC_{50}) was attempted to be determine the toxicity of TCS on this species of crayfish.

Materials and Methods

Materials

Crayfish of the species *Orconectes propinquus* (Figure 2) were collected from local tributaries of the Grand River using a seine net. Twenty-one males and five females were isolated for testing. The Triclosan used in the experiments was purchased from Alfa Aesar in the form of 5-Chloro-2-(2,4-dichlorophenoxy)phenol, 99%. Ethanol was used as a solvent to dissolve the TCS due to its low solubility in water.

Mortality Experiment

Mortality effects of Triclosan on the crayfish were determined by testing exposure to three different concentrations. An initial concentration of 0.35 mg/L, similar the LC_{50} of other aquatic organisms (Adolfsson-Erici, 2002), was set up by dissolving 1.8 mg of TCS in 2 mL of ethanol and 5.14 L of water. Ethanol was selected to help dissolve the TCS into solution as done by Foran et al. in 2000. Four crayfish were placed into a tank with an air stone and the TCS solution and observed every 24 hours for 72-hour time frame. After exposure, all remaining crayfish were placed into an overflow tank with pure water. Subsequent concentrations of 1.0 mg/L and 2.0 mg/L were made in the same manner. Four new crayfish were used for the 1.0 mg/L solution and only one crayfish for the 2.0 mg/L solution. A small number of subjects were used for this test to minimize potential death of crayfish.

Food Localization Experiment

Seven crayfish selected at random were placed into a tank with an air stone and 2.0 mg/L solution of TCS. They were not fed to enhance their desire for food on testing days. Trials took place after 6, 13 and 20 days of exposure. The crayfish were observed every 24 hours for any abnormal activity and to ensure they survived. Crayfish was individually placed into the corner of a 30 cm by 60 cm tank filled with approximately two inches of water to test their ability to find food. One rabbit food pellet (crayfish food used in the lab) was placed in the opposite corner two inches from either wall of the tank. Time was recorded from when the crayfish entered the tank to when they found and began eating the food. If they did not find the food within five minutes they were determined to be unsuccessful. Success rate and average time to find the food for the successful crayfish was recorded. After each set of trials, all crayfish were fed and then placed into a fresh 2.0 mg/L solution of TCS without food.

Attraction to Crayfish Odor Experiments

Sixteen male crayfish were isolated for testing and kept together in a tank with 20 L of solution (Figure 3). Crayfish were tested after exposure to pure water, ethanol and water for 24 hours (vehicle control), and a concentration of 1.5 mg/L of TCS dissolved in ethanol and water after 24 hours, seven days and 14 days. The solution of TCS was made by dissolving 30.0 mg of TCS in 2 mL of 100% ethanol and placed into 20 L of water.

A Y-maze was used as the tank for the trials for each group (Figure 4). The maze was formed from a rectangular, black, acrylic material and the tank measured 152 by 72 by 15

cm. The tank was divided down the middle by a 100 cm long piece of acrylic to create the two arms of the Y-maze measuring 36 cm wide. One arm was for water (neutral) and the other was for water with either male of female crayfish odor. Water flowed into each arm from 1.27 cm diameter clear plastic tubing at a flow rate of 6.15 L/min. The tubing was 237 cm long and connected to a faucet on one end, and then split by a plastic T-connector at the other. Additional plastic tubing, 9 cm in length, connected each arm of this T-connector to additional T-connectors. At each of these connectors, one end served as the injection port for odor and contained a 3 cm long piece of plastic tubing that was plugged with a rubber stopper when not in use. The other end of the connector led to one arm of the Y-maze via a 30 cm long piece of plastic tubing. The inflow holes at the end of each arm measured 0.64 cm in diameter. At the opposite end of the tank, a 1.27 cm hole in the center of the tank served as the drain and drained into a sink. This hole was plugged as the tank filled with water to approximately three inches and then unplugged to create even flow for the experimental trials.

For each trial, 10 of the male crayfish were selected at random and tested individually. Each crayfish was placed into a upside down basket used as a cage Figure 5) located in the middle of the outflow end of the maze for two minutes to allow them to acclimate to the water. Since the flow of odor and non-odor water was split evenly down the middle of the tank (Figure 6), each crayfish had the opportunity to interact with each type of water before being released.

Starting from the moment the crayfish was placed into the tank, 10 mL of water containing either male or female odor was injected into one arm every two minutes using a 60 mL syringe (Figure 7). The scented water was collected from tanks that had contained either 5 males or 5 females in 6 L of pure water for 24 hours (Figure 8). After two minutes of acclimation, crayfish were released from the cage and allowed to explore the maze for 10 minutes. Time in each half of the tank was recorded. After time expired, each crayfish was placed into a separate bucket of water to eliminate the chance of being tested twice. The first five subjects were tested with the odor in one arm, while the next five subjects had the odor in the opposite arm in order to eliminate any bias to one side of the maze. If the crayfish did not explore each arm of the maze at least once, then an additional crayfish was tested who did explore the entire maze. In between changes in which arm the odor was administered and between administration of female and male odor, the previous arm that contained odor was allowed to become neutral for at least 10 minutes before testing the next crayfish with a new odor or odor in the opposite arm.

Results

Mortality Experiment

No crayfish died after 72 hours of exposure to 0.35, 1.0 or 2.0 mg/L of TCS.

Food Localization Experiment

Before being exposed to Triclosan, 57.1% of control crayfish were successful in finding food. Successful crayfish took an average of 42.5 seconds to locate the food. After 6 days of exposure to TCS, 71.4% were successful, taking an average of 40 seconds to find the food. After 13 days of exposure, all of the crayfish were successful at locating the food. An average time of 45.3 seconds was spent locating the source. After 20 days of exposure, 57.10% were successful, taking 28.25 seconds to find the food (Graph 1 & 2). During and after the 20 days, zero crayfish died.

Attraction to Crayfish Odor Experiments

After being exposed to pure water, male crayfish spent 61.02% of the time in the female odor arm and 58.88% in the arm with male odor. After 24 hours of exposure to ethanol and water, 65.2% of the time was spent in the female odor side, while 38.08% of the time was spent in male odor. After 24 hours of exposure to 1.5 mg/L of TCS dissolved with ethanol, crayfish spent 60.46% of the time in female odor and 56.20% in male odor. After 7 days in the TCS solution, only 48.85% of time was spent in female odor and 48.33% in male. After 14 days of exposure to TCS, 52.8% of time was spent in female odor and 48.77% in male (Graph 3 & 4). Days 7 and 14 were significantly different from controls and ethanol controls. During these trials one crayfish died after 9, 11 and 13 days of

exposure. One crayfish also died 2 days after being removed from the TCS solution, 16 days after initial exposure began. A total of 4 crayfish died for a 25% mortality rate.

Discussion

Compared to other aquatic organisms, *Orconectes propinquus* appears to have a significantly higher tolerance for TCS. A LC_{50} was unapparent at concentrations more than five times higher than other aquatic organisms (Adolfsson-Erici, 2002). Although a higher tolerance is observed, TCS still shows harmful effects towards the species. Results indicate that TCS is not immediately toxic to crayfish, though, even at concentrations three times higher than reported in the environment. Instead TCS seems to have an accumulation effect, taking around a week to build up in the body and cause any effects. In experiments looking specifically for an LC_{50}, no crayfish died within the first 72 hours. However, a death of a crayfish was observed in the Y-maze trials after 9 days of exposure to 1.5 mg/L of TCS. Subsequent deaths occurred after 11 and 13 days of exposure, and one additional death occurred 2 days after the crayfish were taken out of TCS. The last death is indicative that TCS may accumulate and reside in the body, causing post-exposure effects. A lethal stress response may also be occurring after exposure. We propose further examination of crayfish eggs and juvenile crayfish that may be more susceptible to such a chemical when compared to adults.

TCS appears to show some effects on physiological systems in crayfish, but olfaction towards a food source does not seem to be one of those systems. Over the course of the 20-day exposure, the success rate of the crayfish finding the food source actually increased after six and 13 days, but was equal to the control group at 20 days. The increasing fluctuations in the first half of the exposure is likely due to chance, since there was such a small sample size of only seven crayfish. Furthermore, the average time for successful

crayfish to locate the food did not vary much, ranging from only 40-45 seconds from controls to 13 days of exposure. Although successful crayfish took an average of only 28 seconds after 20 days of exposure, this could again be due to the small sample size. The fact that success rate was unchanged after 20 days and average time to find food was consistent gives evidence that TCS does not affect the ability or desire of crayfish to find food, but a larger sample size is needed to declare this without doubt.

In agreement with previous reports, TCS has shown to disrupt endocrine systems in *Orconectes propinquus.* Before exposure to TCS, male crayfish exhibited an attraction to female odor, spending between 61-65% of their time in the female odor arm over a neutral odor arm of the Y-maze. Even after 24 hours of exposure, the male crayfish still spent 60% of their time in the female odor. The absence of difference after 24 hours of exposure is evidence that TCS does not have immediate effects to the animals. However, after 7 days of exposure, time spent in female odor went down to only 49%, and after 14 days only 53% of time was spent in female odor. The male crayfish previously showed an attraction towards females, but after being exposed to TCS for several days the time spent in either arm of the Y-maze was essentially random, showing no preference towards females. We believe the food and female odor trials support the notion that TCS does not alter olfactory abilities. Instead we propose that TCS is affecting the endocrine system of the crayfish, thus affecting either the males' ability or desire to find a female mate. Disrupting this attraction towards female crayfish in the environment can have detrimental effects to the species. By decreasing a male's attraction towards females, the ability of the

species to reproduce would be greatly reduced. Over time, this could lower the population of the species and have dire consequences to aquatic food webs.

Although TCS seems to decrease the attraction of male crayfish towards females, there does not appear to be a consistent effect on their attraction towards conspecific males. The time spent in the male odor fluctuated up and down for each exposure group, but throughout the duration of the experiment the subjects spent close to 50% of their time in the odor. With the crayfish spending close to half of their time in the male odor both before and after exposure to TCS, the crayfish explored each arm of the tank at random. There does not seem to be an attraction or repulsion towards other males after exposure to TCS, which may not change the frequency of social interactions in nature between male *Orconectes propinquus.* However, we recognize that there may be a change in the aggression exhibited by males and have designed a future study to test agonistic behavior of crayfish after exposure to TCS to examine for such an effect.

The effects of this study give further evidence to question the use of TCS in consumer products. The harmful effects to *Orconectes propinquus* from endocrine disruption to death will negatively impact the population size of a key organism in nature over time. As use of TCS continues and grows, environmental concentrations will also continue to rise. Effects will only be amplified as this occurs. As TCS levels increase and continue to accumulate in aquatic organisms, the chemical will increasingly make its way up the food chain into humans as it has already been recorded (Burton, 2012). Effects in humans are not known yet, but based on previous findings and findings in this study, endocrine

disruption may be of concern. With current and previous research results, we recommend this widely used antimicrobial compound that offers little to no benefits over plain soaps should be removed from consumer products for the safety of both humans and other animals in the environment.

In order to collect more evidence for the discontinuation of TCS, future research could target different aspects in *Orconectes propinquus.* Exposures to eggs, hatchlings or juvenile crayfish could be done to discover toxicity or developmental effects due to TCS's endocrine disruption mechanisms. To continue off of these experiments, larger sample sizes could also be used as well as tests at lower concentrations closer to those last reported in the environment.

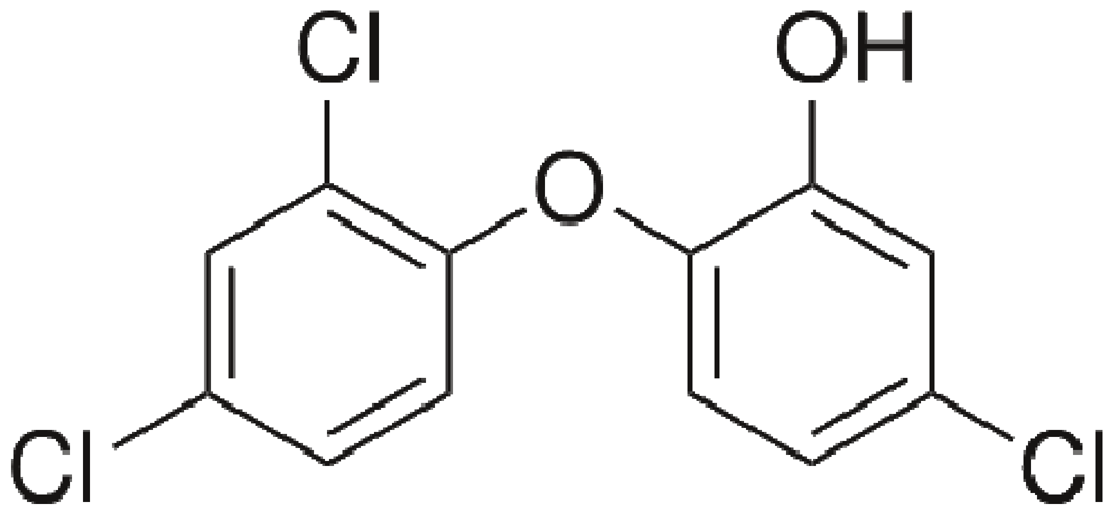


Figure 1. Chemical structure of Triclosan 5-chloro-2-(2,4-dichlorophenoxy)phenol

Figure 2. *Orconectes propinquus* crayfish

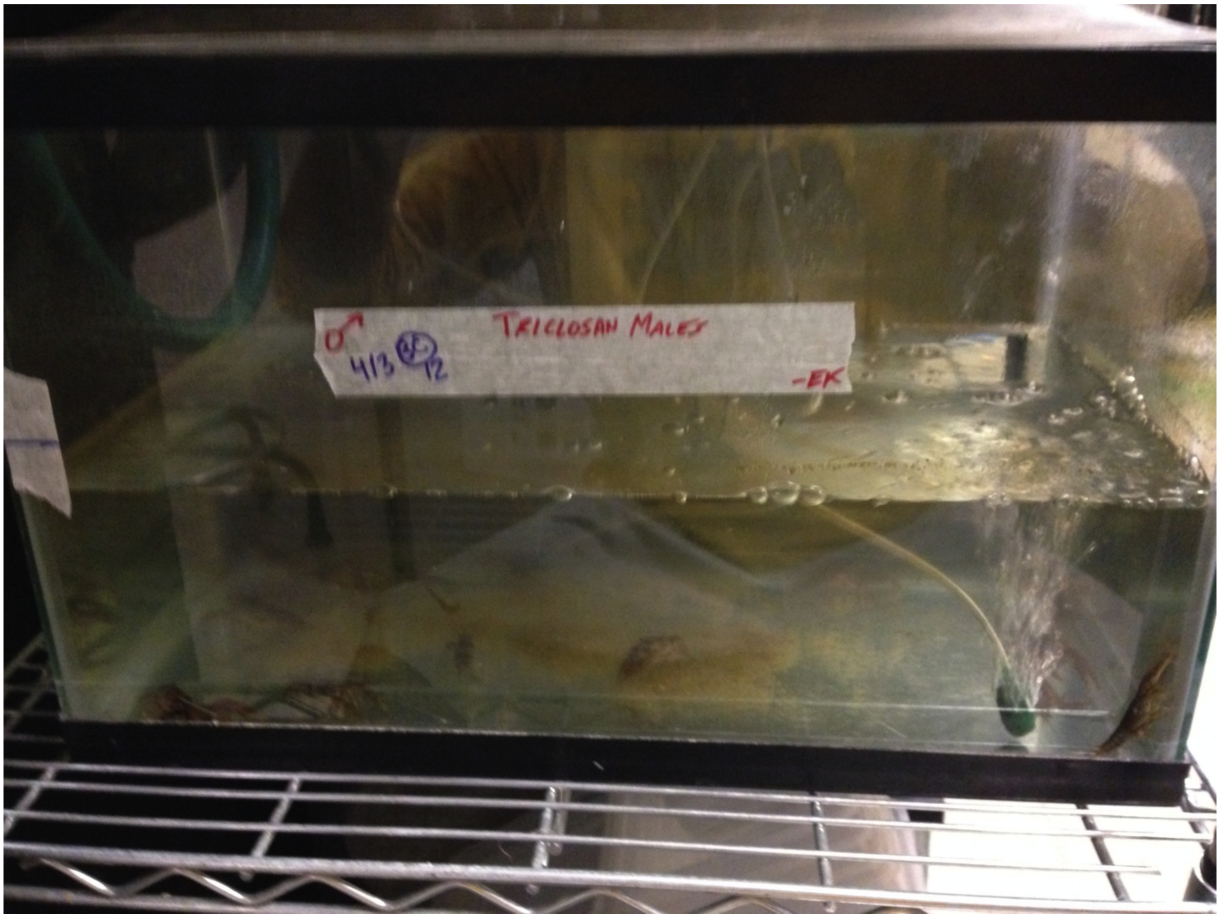


Figure 3. Male crayfish housed for Y-maze trials.

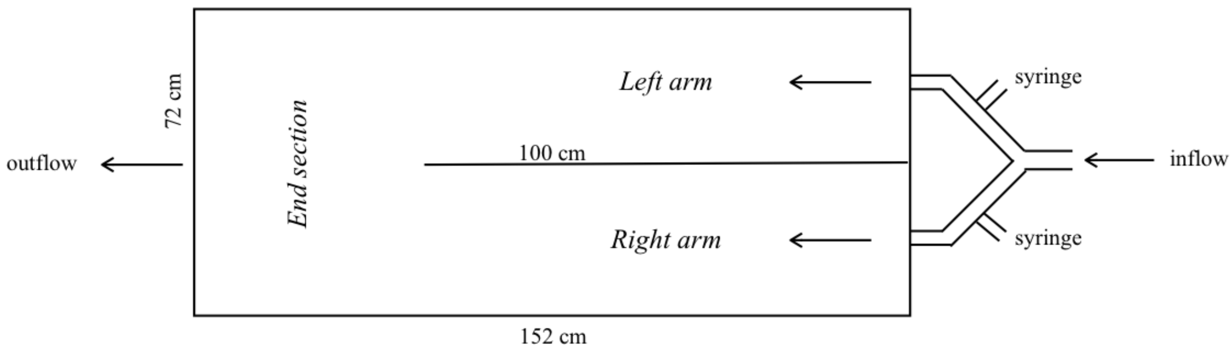


Figure 4. Setup of Y-maze

Figure 5. Crayfish held in basket to allow for acclimation to surroundings in the Y-maze.

Figure 6. Demonstration using food coloring to show the flow of water in the Y-maze split evenly into two arms.

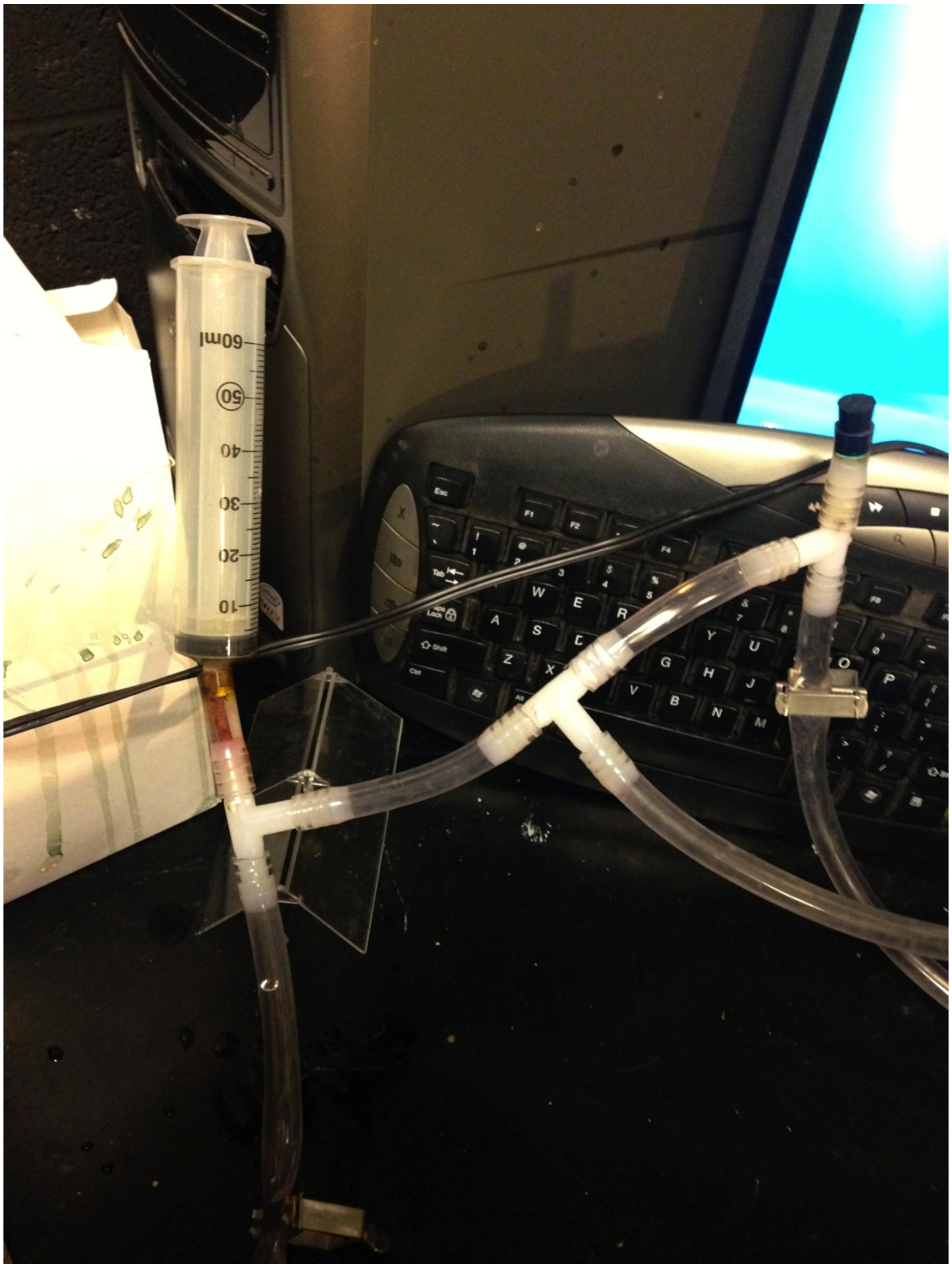
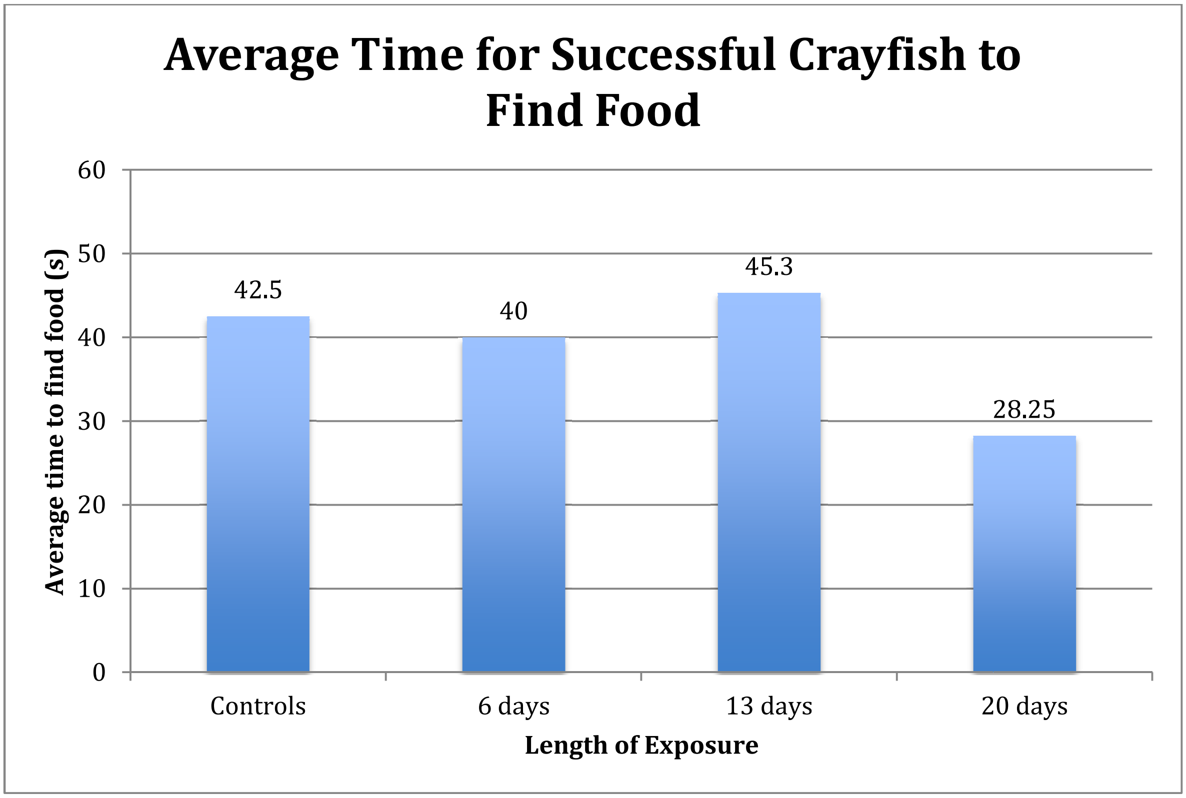
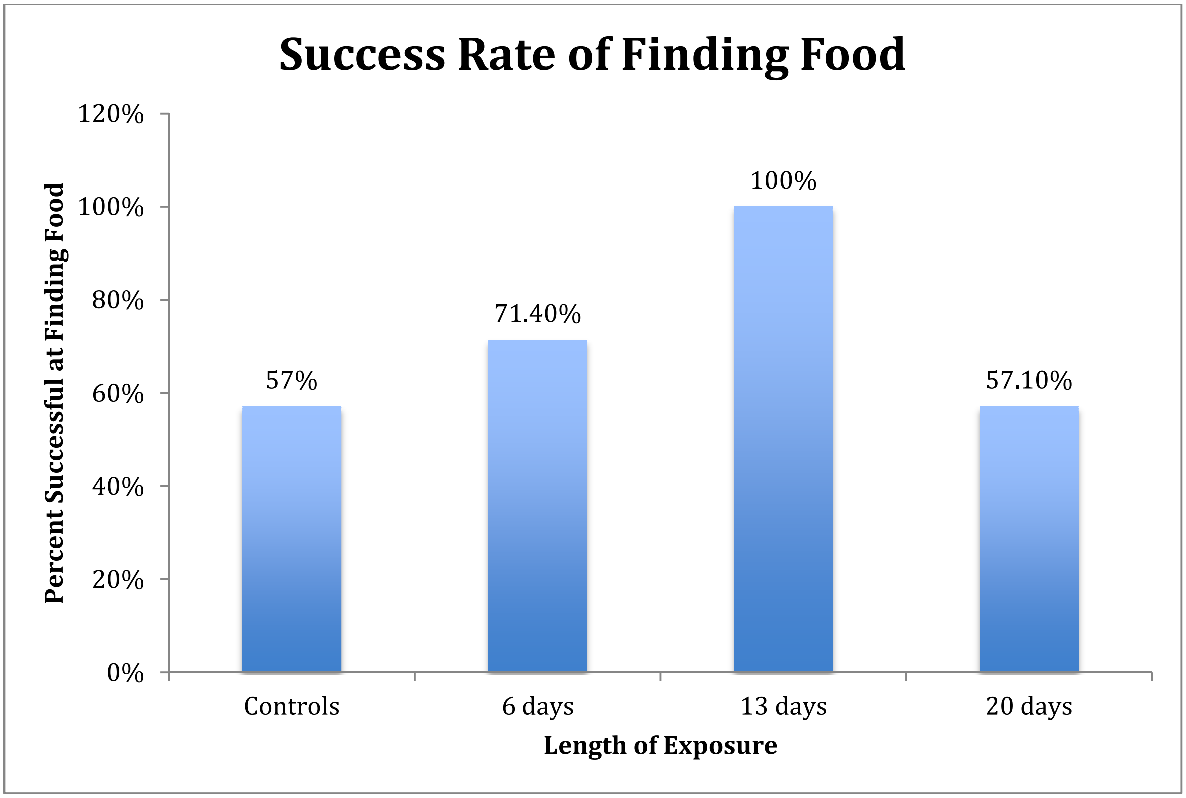


Figure 7. Syringe used to inject odor into Y-maze.

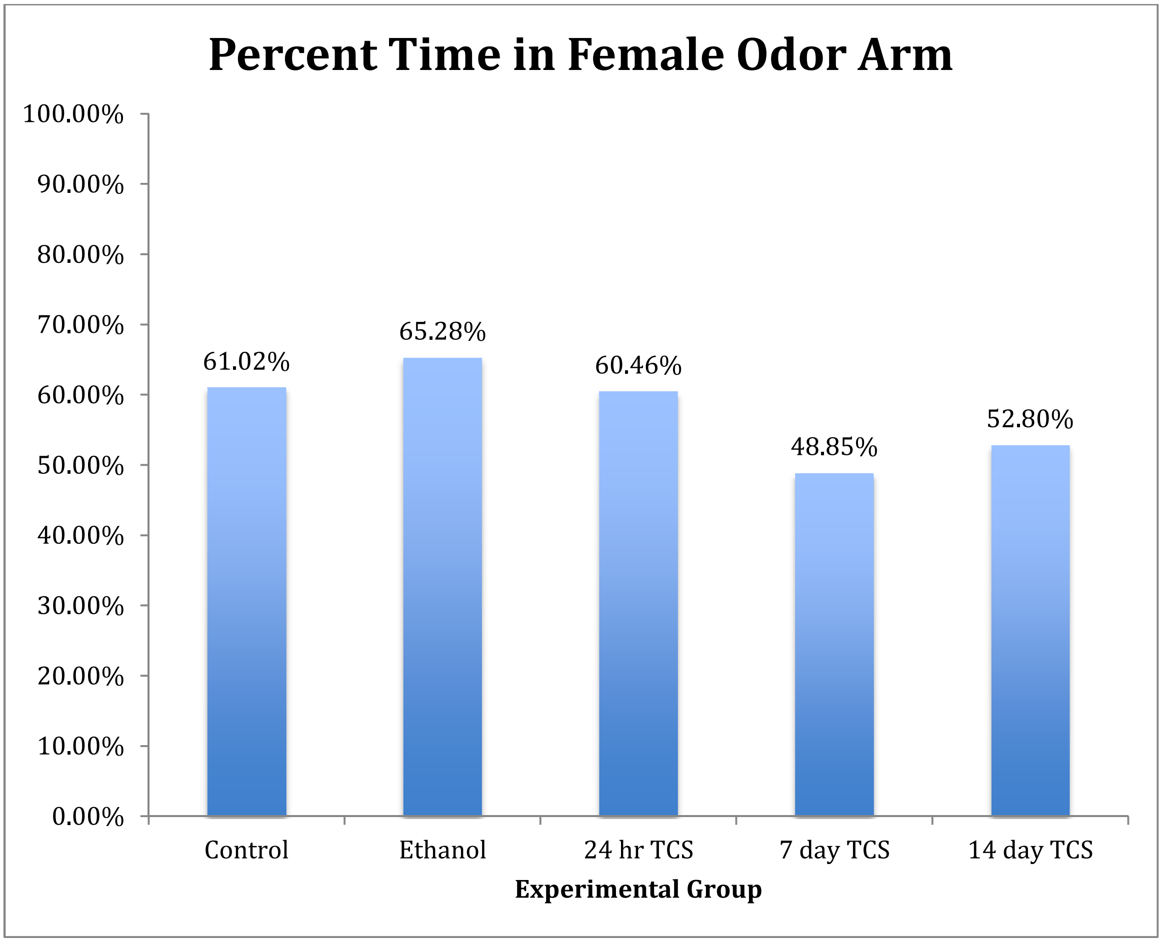
Figure 8. Male and female crayfish used to collect odor.


Average Time for Successful Crayfish to Find Food
Average time to find food (s)
60
50
40
30
20
10
0
42.5
40
45.3
28.25
Controls
6 days
13 days
20 days
Length of Exposure

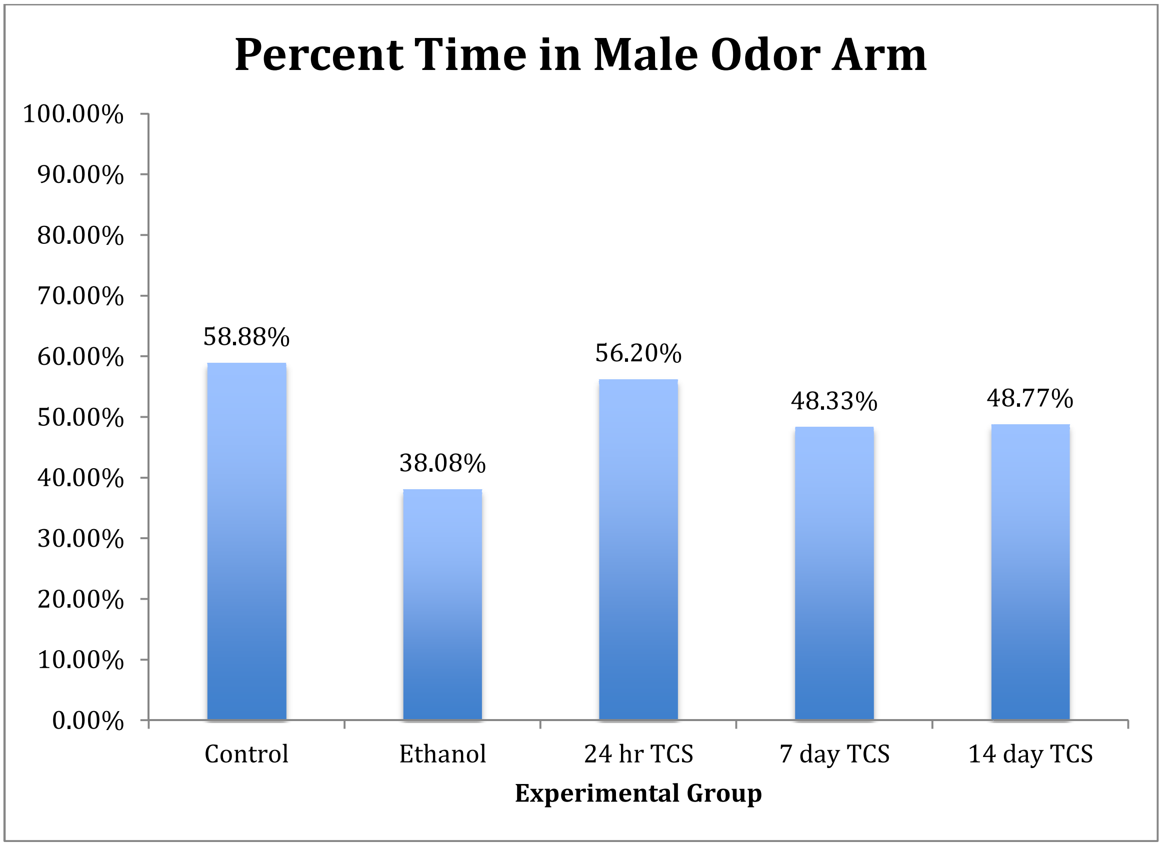
Graph 1.


Success Rate of Finding Food
120%
100%
80%
60%
40%
20%
0%
Percent Successful at Finding Food
57%
71.40%
100%
57.10%
Controls
6 days
13 days
20 days
Length of Exposure

Graph 2.


Percent Time in Female Odor Arm
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
61.02%
65.28%
60.46%
48.85%
52.80%
Control
Ethanol
24 hr TCS
7 day TCS
14 day TCS
Experimental Group

Graph 3.


Percent Time in Male Odor Arm
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
58.88%
38.08%
56.20%
48.33%
48.77%
Control
Ethanol
24 hr TCS
7 day TCS
14 day TCS
Experimental Group

Graph 4.

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