THE EFFECTS OF SUB-LETHAL LEVELS OF 2,4-DICHLOROPHENOXYACETIC ACID HERBICIDE ON FORAGING BEHAVIORS IN THE CRAYFISH, ORCONECTES RUSTICUS

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ABSTRACT

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The widespread use of herbicides across the globe has increased the probability of synthetic chemicals entering freshwater habitats. Upon entering aquatic habitats, these chemicals target and disrupt both physiological and behavioral functioning in various aquatic organisms. Herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D), can have negative impacts on chemoreception because these receptor cells are in direct contact with water-soluble chemicals in the environment. Studies focusing on lethal concentrations (LC50 levels) may understate the impact of herbicides within aquatic habitats as damage to the chemoreceptors can result in modified behaviors or lack of appropriate responses to environmental or social cues. The purpose of this experiment was to determine whether exposure to sub-lethal levels of 2,4-D alters the foraging behaviors of crayfish, *Orconectes rusticus*. We hypothesized that crayfish exposed to greater concentrations of 2,4-D would be less successful in locating food, or upon locating food, would consume smaller amounts possibly due to an inability to recognize the food odors in the contaminated waters. Crayfish were exposed to three sub-lethal levels of 2,4-D for 96 h and placed into a Y-maze system with a fish gelatin food source placed randomly in the right or left arm. Average walking speed, average time spent in the correct arm, and percent consumption were analyzed. Our data show crayfish were impaired in their ability to forage effectively. These inabilities to locate and consume adequate amounts of food could result in lower body weights and decreased fitness in the populations of crayfish exposed to 2,4-D in natural habitats.
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INTRODUCTION

Herbicides are used in the Midwestern United States as a means to control weed production and increase crop yield. 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most widely used herbicide in the world (Wauchope et al. 1992; Teixeira et al. 2007), with over 1,500 herbicide and pesticide products on the market that contain 2,4-D as the primary active ingredient (Howard 1991; U.S. EPA 2002). The increased popularity of the herbicide in both urban and agricultural landscapes increases the probability of 2,4-D entering freshwater habitats through precipitation or direct application. Upon reaching a stream or body of water, pesticides such as 2,4-D can enter an organism’s body through consumption, respiration or absorption through the skin (Helfrich et al. 2009) causing behavioral and physiological changes.

Deleterious physiological and behavioral consequences have been reported in experiments testing the effects of 2,4-D exposure on aquatic organisms. Farah et al. (2004) found that several species of fish (*Heteropneustes fossilis*, *Clarias batrachus*, *Channa punctatus*) exposed to 2,4-D displayed stress behaviors such as abnormal and restless swimming, vigorous jerks of the body, anorexia and loss of balance. Similar results were found in Nile tilapia larvae and adults, where exposure to 2,4-D resulted in erratic swimming and respiratory difficulties (Sarikaya and Selvi 2005). The presence of 2,4-D on the Brazilian pearl eartheater (*Geophagus brasiliensis*) resulted in reduced oxygen consumption and ammonium excretion (Barbieri 2009). Especially in the case of direct aquatic applications, the concentrations of 2,4-D can be well above the LC50 levels for numerous aquatic species. These high concentrations will negatively affect physiology and behavior, particularly those behaviors influenced by the chemoreception.

Contamination within an aquatic ecosystem caused by herbicides is believed to have particularly harsh effects on the olfactory system because the olfactory sensors are in direct
contact with the water-soluble stimuli in the environment (Moore and Waring 1996; Scholz et al. 2000; McPherson et al. 2004). Chemoreception in fish and crustaceans is responsible for ecologically important behaviors, such as courtship rituals, foraging for food, orientation towards long distance stimuli, and learning (Atema 1977; 1995; Caprio and Derby 2008). Damage to the olfactory receptors due to pollutants can result in modified behaviors or lack of appropriate responses to environmental or social cues (Olsén 2011). Pollutants have been shown to disturb the proper binding of the female pheromone to the male olfactory receptors in brown trout, resulting in a lack of attraction of the male to the female, leading to potential effects on the courting events and reproduction within the community (Jaensson and Olsén 2010; Olsén 2011). Reduction in both pheromone release and detection was also observed in salmon exposed to the synthetic pesticides atrazine, diazinon and Cypermethrin (Moore and Waring 1996; 1998; 2001). An impairment of the chemoreception in adult salmonid fish may also diminish their homing ability to identify and return to their native river to spawn (Hasler and Scholz 1983; Bertmar 1982). Sherba et al. 2000 observed that individuals of freshwater crayfish, Cambarus bartonii, were unable to find food in a Y-maze when exposed to low concentrations of copper. Although the majority of studies regarding pollutants in aquatic habitats have focused primarily on behavior in fish, any animal that relies on chemoreception for foraging, orientation or social behaviors is threatened by exposure to unnatural levels of metal ions, pesticides, or synthetic chemicals present in their ecosystems.

Crayfish are model animals for the analysis of sub-lethal effects of herbicides on chemoreception. Disruption in functional chemoreceptors can significantly inhibit proper behavior (Klaprat et al. 1992; Olsén 2011). These animals rely on chemical stimuli for identifying mates and predators (Hazlett, 1990; Keller and Moore 1999; Giri and Dunham 2000;
Belanger and Moore 2006), determining social status (Zulandt Schneider et al. 2001), and locating food (Moore et al. 1991). A previous study by Wolf and Moore (2002) have indicated that sub-lethal levels of the pesticide metolachlor interfere with chemically-mediated foraging behaviors in crayfish, *Orconectes rusticus*. Considering their position as critical species in aquatic systems and reliance on chemoreception for behavior, crayfish can be used as indicators of stream health and water quality.

Crayfish are benthic omnivores that can be found in diverse aquatic habitats, ranging from lakes to rivers and streams, and are of great ecological importance. With a diet consisting of a variety of aquatic species, crayfish have significant and simultaneous impacts on multiple trophic levels present in their habitats (Lorman, 1975; Lodge et al. 1994). These aquatic animals are also critical in the processing of organic matter present in rivers and streams (Usio and Townsend 2002). The addition or removal of crayfish into freshwater ecosystems has resulted in observed changes in the abundance of plankton and fish populations, dissolved oxygen level, and biomass of macrophytes (Dorn and Wojdak 2004). Because they have significant impacts on various trophic levels and nutrient cycling, crayfish are considered highly important species in their habitats. Their role as central species in their habitats means their survival is critical for the health and development of freshwater ecosystems.

In previous studies, the acute toxicity of 2,4-D on crayfish was determined, but the investigation of sub-lethal exposures on crayfish behaviors was still lacking (Benli et al. 2007). The purpose of this experiment was to determine whether exposure to sub-lethal levels of 2,4-D alters the foraging behaviors of crayfish, *Orconectes rusticus*. We hypothesized that crayfish exposed to greater concentrations of 2,4-D would be less successful in locating food. Furthermore, we hypothesized that should crayfish find the food, they would consume smaller
amounts of food, possibly due to the inability to recognize food odors in the contaminated waters.
MATERIALS AND METHODS

Animals

Male and female crayfish, *Orconectes rusticus*, were collected from the Portage River near Bowling Green, Ohio (Wood County, 41°21’42”N, 83°35’28”W). All crayfish were sized and sexed. Only crayfish with intact appendages were used in this study. Crayfish were mechanically and visually isolated in a flow through tank within an environmental chamber at a constant temperature (23°C) and light/dark cycle (12L:12D). Crayfish were 2.5 ± 0.2 cm in carapace length. All crayfish were fed a fish gelatin food source and then starved for one week prior to trials in order to increase motivation for foraging behavior. Crayfish were used only once in these trials.

Chemical and Stimuli Preparation

To test for any behavioral impairments due to exposure to herbicide, we isolated crayfish into exposure tanks and performed an acute exposure to 2,4-D. 2,4-D [(2,4-dichlorophenoxy)acetic acid; CAS Number: 94-75-7] was purchased from the Sigma-Aldrich Company, Saint Louis, MO. Stock solutions (1 g/10 mL 2,4-D) were made and stored in the dark at 4°C.

Crayfish were exposed three at a time to each treatment for 96 h in 97 L (34 x 94 x 30.5 cm) exposure tanks before testing in the foraging assessments. Each exposure tank had a gravel substrate identical to the Y-maze arena and three shelters to reduce aggression through competition of shelter. Each exposure cycle was initiated with fresh tank water and 2,4-D solutions.

Fish gelatin was prepared as a food source for the behavior analysis. The food was prepared by mixing 45 g of sardines with 28 g of unflavored gelatin (Knox®) and 0.71 L of
boiling water in a blender until homogenized. After mixing, the gelatin was placed into plastic caps for added weight, covered with plastic wrap and refrigerated overnight until solidified. Each cap contained 5g of food and fish gelatin was not used longer than 72 h after preparation. Crayfish were fed fish gelatin for two weeks prior to trials, allowing them to immediately recognize the gelatin as a food source.

Experimental Design

Crayfish were exposed for 96 h to three sub-lethal concentrations of 2,4-D: 32.69 mg/L (high), 14.07 mg/L (medium) and 7.65 mg/L (low). Concentrations were chosen based on an exponential regression run on the acute 96 h toxicity of 2,4-D concentrations on crayfish survival (Benli et al., 2007).

Exposure treatments were as follows:

**Treatment 1**, LC40 (high), \(n=15\): 32.69 mg/L (16.02 mL 2,4-D stock: 49 L of dechlorinated tank water)

**Treatment 2**, LC20 (medium), \(n=15\): 14.07 mg/L (6.89 mL 2,4-D stock: 49 L of dechlorinated tank water)

**Treatment 3**, LC10 (low), \(n=15\): 7.65 mg/L (3.75 mL 2,4-D stock: 49 L of dechlorinated tank water)

**Treatment 4**, Control Treatment, \(n=19\): 49 L dechlorinated tank water only

Y-Maze Design

A flow-through Y-maze was used to test crayfish response to food odor in the presence of 2,4-D concentrations (tank= 76 x 40 x 30 cm, arm= 50 x 20 x 30 cm; Fig 1). Two elevated reservoir tanks (4.45 L) supplied water and 2,4-D stock solution to the arms of the maze through 12.7 mm (ID) Nalgene® tubing. Two in-line flowmeters (Monostat Riteflow #3) were used to
control the flow into the maze. Water exited the system at the opposite end from the chemical entry point through five outflow pipes that were 5 cm above the bottom of the maze.

The Y-maze was divided into three of zones to assess the amount of time spent in each region of the maze (Fig 1). The arms of the maze were separated by a partition and the arm was defined as the back of the maze near the odor source to the end of the partition, 1,000cm$^2$ in area. The remaining end of the maze was a neutral zone, in which crayfish were considered not in either arm of the maze. Walls and partition of the maze were opaque and black in color.

**Experimental Protocol**

Crayfish foraging behaviors were assessed in the same 2,4-D concentrations that they were exposed to for 96 h to best represent the persistence of the herbicide in a natural aquatic system. Food placement in the Y-maze was assigned randomly into the left or right arm in each trial by flipping a coin. Food was placed 3cm in front of the nozzle in the left or right arm. Crayfish were acclimated in a gated shelter for 10 min while the flow was initialized. After the acclimation, the shelter was removed and crayfish were allowed to explore the tank for 15 min with a constant flow rate of 50 mL/min. At the end of the 15 min, crayfish were placed back in the gated shelter as the food was placed into the system. Once the food was placed in the appropriate arm of the maze, crayfish were released and the trial time began. The trial ran for 15 min in length and continued even if they crayfish found the food source. Any trials where the animal tailflipped immediately after the shelter was raised were excluded because this behavior was an abnormal reaction due to external stimuli. Trials were recorded digitally with a camera (Panasonic HDC-HS250) and analyzed on an HP Pavilion g6 Series laptop computer. The Y-maze and gravel were rinsed for 10 min with alternating hot and distilled water between trials in which different exposure concentrations were used.
Data and Statistical Analysis

Video recordings were analyzed to determine average walking speed and time spent in each zone by using EthoVision XT 8.5 software (Noldus Information Technology, Wageningen, The Netherlands). Crayfish were considered in or out of a zone or arm of the maze when its rostrum passed a line separating the divisions (see Fig. 1 for divisions).

The time spent in each zone was determined using the EthoVision XT 8.5 software (Noldus Information Technology, Wageningen, The Netherlands). The amount of time a crayfish took to find the food was determined by calculating the length of time required for the crayfish to leave the shelter and directly touch the food source with at least one chela while facing the food source. The orientation parameters calculated using EthoVision XT 8.5 software (Noldus Information Technology, Wageningen, The Netherlands) included average walking speed, average time spent in the correct arm, and average distance to the source. Consumption was calculated by subtracting the weight of the food source after the trial from the weight of the food source prior to the trial. Percent consumption was calculated by dividing the weight of the consumption by the weight of the initial food source before the trial began. Since the gelatin food source absorbed water throughout the trial period, weight of the gelatins after the trial were normalized to the original weight prior to consumption.

Average walking speed, average time spent in the correct arm, final distance from the source and percent consumption were analyzed using a one-way multivariate analysis of variance (MANOVA) with Fisher least significant differences (LSD) post hoc analysis. All data were analyzed using Statistica 6.0 (one-way MANOVA, StatSoft, Tulsa, OK) and Microsoft Excel 2007 with significance set at p-values less than 0.05.
RESULTS

Time to Locate Food

There was an overall significant effect of 2,4-D on crayfish food finding behavior ($F_{(18,156, 0.05)}=3.09$). Crayfish exposed to high concentrations of 2,4-D took significantly longer to locate the food source when compared to control animals and those exposed to medium concentrations (Fisher-LSD post hoc; $p < 0.05$). Crayfish in the control group took a mean of $256.6 \pm 49.8$ (SEM) s to locate the food, which was significantly different from the highest exposed group ($559.6 \pm 87.9$ s). The times to locate the fish gelatin for the highest exposed experimental group were also significantly different from the times to locate food for the medium exposed group ($315.2 \pm 67.6$ s). The low exposed crayfish were not significantly different from other test groups ($432.5 \pm 85.9$ s) (Fig. 2). Of the crayfish tested, 18 of the 19 controls, 11 of 15 low exposed, 14 of 15 medium exposed and 10 of 15 highly exposed crayfish successfully located the food sources. These results were not significantly different.

Time Spent in Correct Arm

Crayfish exposed to 2,4-D herbicide spent less time in the arm of the maze that contained the food source (Fig. 3). The majority of the trial period for exposed crayfish was spent in the incorrect arm or in the neutral zone at the end of the maze. Crayfish exposed to 2,4-D spent significantly less time in the correct arm of the maze than did the controls, and the times for each exposed group were not significantly different from one another, low=$314.8 \pm 37.0$ s, medium=$309.3 \pm 55.2$ s, and high=$301.3 \pm 59.5$ s (MANOVA; $p < 0.05$). Control crayfish spent significantly more time in the correct arm of the maze than the herbicide exposed crayfish, spending a mean of $487.9 \pm 48.1$ s in the arm with the food source.

Walking Speed
Analysis indicated an overall treatment effect on the walking speeds due to exposure to low and medium 2,4-D exposure conditions (MANOVA; p < 0.05 (Fig. 4)). Walking speeds were determined only by analyzing the times crayfish were in motion. Crayfish exposed to low and medium concentrations of 2,4-D had significantly faster walking speeds than the controls who had a mean speed of 2.1 ± 0.1 cm/s. Exposure to low levels resulted in the highest walking speed across all treatment groups with a velocity of 3.0 ± 0.1 cm/s. As concentration of herbicide increased, the walking speeds of the crayfish decreased.

**Overall Speed**

Overall speed was fastest for animals in the presence of 2,4-D than in the control treatments (Fig. 5). The control crayfish had the slowest overall speed, with a mean speed of 1.2 ± 0.1 cm/s. This speed was significantly different from both the low exposure (1.9 ± 0.1 cm/s) and medium exposure (1.6 ± 0.2 cm/s) treatment groups, but not significantly different from the high exposure group (1.2 ± 0.1 cm/s) (MANOVA; p < 0.05).

**Percent Consumption**

Crayfish exposed to all concentrations of herbicide demonstrated a diminished percent consumption of the fish gelatin food sources (Fig. 6). The high exposure treatment group had a total percent consumption rate of -2.4 ± 2.9 %, meaning that their gelatin food source gained more mass through water absorption than the crayfish consumed, if consumption did occur. All exposed crayfish consumed significantly less food than the control group with a mean of 14.4 ± 3.3 % (MANOVA; p < 0.05).
**Final Distance from Source**

All trials resulted in a variance of final distances from the food source, regardless of treatment. Animals that located the food did not stay near the source until the end of the trial. Overall, the statistical analysis did not show any difference between treatment groups ($p > 0.05$).

**Initial Arm Choice**

Initial arm choice across each treatment was not significantly different (Tukey Multiple Proportions, $p > 0.05$). Eleven of the 19 control crayfish entered first into the correct arm of the maze, compared to 6 out of 15 in the low treatment, 9 out of 15 in the medium and only 5 out of 15 in the high treatment group.
DISCUSSION

The results of this study indicate that sub-lethal levels of the herbicide 2,4-D affect a chemically mediated behavior of crayfish. 2,4-D inhibits the ability of crayfish to locate food by causing crayfish to walk at more rapid speeds (Fig. 4 and Fig. 5), spend less time in the correct arm of the maze (Fig. 3) and take significantly longer to locate food than the control crayfish (Fig. 2). The longer amount of time it took crayfish to find food sources (Fig. 2), the less time remained for consumption (Fig. 6). Taken together these results show that an important chemically-mediated behavior is significantly impaired by exposure to sub-lethal levels of 2,4-D. These impairments were evident in every exposed treatment group, indicating that low amounts of 2,4-D in the environment can have deleterious impacts on crayfish foraging behaviors.

Crayfish rely on spatial information extracted from chemical signals in order to successfully orient to or away from a chemical source (Yakovlev et al. 1974; Atema 1996; Moore and Grills 1999; Webster 2000; Keller et al. 2001; Tomb et al 2001; Fedotov 2009). In addition to the foraging behaviors discussed in this study, chemoreception is also required for identifying conspecifics (Hazlett 1990), locating mates (Giri and Dunham 2000; Belanger and Moore 2006), recognizing predators (Keller and Moore 1999) and determining social status (Zulandt Schneider et al. 2001). The detection of sex pheromones is vital in the reproductive behaviors of crayfish (Ameyaw-Akumji and Hazlett 1975). If chemoreception is inhibited in crayfish exposed to 2,4-D, the structure of social hierarchies will suffer because crayfish will no longer be able to identify social status via urine excretions (Cook and Moore 2008). This inability to detect social status will reduce the likeliness of exposed crayfish to initiate fights (Cook and Moore 2008) and fights could reach higher intensity levels and last for longer periods of time (Zulandt-Schneider et al. 2001). The obstruction of chemical signal detection in crayfish
can ultimately cause a reduction in appropriate response to mating cues, predator signals and an inability to identify social status.

Crayfish rely significantly on their ability to detect odor cues in the environment to make important ecological decisions (Ameyaw-Akumfi and Hazlett 1975; Keller and Moore 1999; Giri and Dunham 2000). If external chemoreceptors are impaired, crayfish would be incapable of properly detecting food odor signals. As a consequence, crayfish would be unable to locate food sources, potentially leading to starvation and the eventual death of the individual (Pesenko and Belyanin 1980; Kraus-Epley and Moore 2002; Fedotov 2009). Crayfish in polluted systems take significantly longer to locate food than crayfish from non-polluted environments (Sherba et al. 2000; Wolf and Moore 2002). The increased amount of time required to successfully find one source of food means a greater energy expenditure during foraging, as well as increased exposure to predation risks. Devoting more time to the detection and location of food will cause a direct reduction in the amount of time being allocated to other essential behaviors such as reproduction and predator avoidance (McNamara and Houston 1986; Ludwig and Rowe 1990; Anholt and Werner 1995 (tadpoles); Biro et al. 2003; Kotler et al. 2004 (gerbils)). The higher walking speeds coupled with lower consumption and reduced success at finding foods means a much higher rate of energy loss for impaired crayfish. These consequences result in less energy resources available for essential behaviors such as mating, foraging, growth and escaping predation (Alexander 1967; Rowe et al. 2000).

In addition to extra energy expenditure, faster walking speeds can negatively affect extractions of information from the environment. Numerous studies have shown that crustaceans reduce walking speeds during chemically-mediated behaviors (Moore et al. 1991; Atema 1996; Grasso et al. 1998; Moore and Grills 1999). One explanation of this chemically-mediated
orientation behavior is that the chaotic nature of odor plumes requires aquatic animals to slow
their walking speeds in order to extract relevant spatial information (Atema 1996; Grasso et al.
1998; Moore and Grills 1999). The higher walking speeds and overall speeds of the 2,4-D
exposed crayfish in this study are similar to the walking behaviors of crayfish and lobsters that
are unable to detect odor signals in their surroundings. The crayfish exposed to the highest
concentrations of 2,4-D exhibited a lack of walking speeds and overall speeds, spending the
majority of the trial time in the incorrect arm or neutral zone of the arena. This absence of
locomotion and reduction in speed has been observed in other studies that exposed aquatic
animals to higher concentrations of pollutants (Atchison et al. 1987; Kavitha and Rao 2007). Our
results indicate that the ability of crayfish to properly sample and orient towards higher
concentrated, close proximity odor signals is significantly reduced. The inability to locate odors
has consequences beyond foraging as crayfish use chemical signals for predator recognition,
identification of alarm cues and conspecifics, dominance hierarchies, and mating (Hazlett 1990;
Zulandt Schneider et al. 2001; Belanger and Moore 2009). Furthermore, younger life stages and
post-molt stages face greater mortality rates during exposure to pollutants due to increased
sensitivity to chemicals (Berrill et al. 1985; Naqvi and Flagge 1990; Wigginton and Birge 2006).

Environmental toxins have been shown to have negative effects on the chemoreceptors of
aquatic animals. Exposure to manganese and crude oil caused structural damage to the
chemoreceptors in lobsters (Atema and Stein 1974; Krång and Rosenqvist 2006). The herbicide
diazinon also been shown to decrease the antennular sensitivity of crayfish to hydrodynamic
signals present in the water, presumably due to physiological damage to receptor cells
(Monteclaro et al. 2011). Food odors can further be blocked or masked by environmental
pollutants adhering to the chemoreceptors, decreasing the likelihood that the odor will be
perceived in the contaminated surroundings (Blumer et al. 1971; Tierney et al. 2010; Blinova 2012).

Other studies using anthropogenic chemicals have demonstrated similar deficits in chemically-mediated behaviors in other aquatic organisms (Beauvais et al. 2000; Brewer et al. 2001; Wolf and Moore 2002; Baldwin et al. 2009; Frontera and Vatnick 2011). Given the prevalence of chemoreception in aquatic animals, 2,4-D most likely will have negative impacts on other ecologically important aquatic animals. Aquatic animals use chemical signals to perform homing to streams for reproduction (Hasler and Scholz 1983), mate identification (Moore and Waring 1996; 1998; 2001) and food location (Sherba et al. 2000). Significant and wide spread pollution in habitats can have drastic consequences for a wide range of aquatic species (Saglio et al. 2003; McPherson et al. 2004; Krång and Ekerholm 2006; Jaensson and Olsén 2010).

In addition to alterations in chemically-mediated behavior, negative impacts on physiological functioning due to pollution exposure have also been found in fish species: reduction in growth rates (Brazner and Kilne 1990; Baldwin 2009; Frontera and Vatnick 2011), hormone production (Moore and Waring 1996), and DNA damage (Lowcock et al. 1997; Whitehead et al. 2004). Sub-lethal exposure to the glyphosate-based herbicide Roundup® caused freshwater fish (Channa punctatus) to exhibit an inhibition of antioxidants in the gills and experience chemical-induced oxidative stress (Nwani et al. 2013). To counter the harmful effects of contamination, fish placed in habitats with environmental and chemical stressors were shown to allocate energy to maintenance from production activities (Calow 1999; Handy et al. 1999). The results from our study serve as an indication that the survival of aquatic species that rely on chemical signaling for appropriate behavior is jeopardized by the presence of 2,4-D at sub-lethal
levels, and that numerous aquatic species could be impacted by the exposure behaviorally and physiologically (Weis et al. 2001; Mills and Semlitsch 2004).

If exposure to 2,4-D has significant impact on key aquatic species, such as crayfish, any changes to the aquatic food web structure could have broad ranging impacts to the populations of many other organisms. Serving as keystone species in freshwater systems, several species rely on crayfish for food. Crayfish also help to control the abundance of algae and invertebrates through predation and consumption, while contributing to nutrient cycling and dissolved oxygen levels (Creed 1994; Dom and Wojdak 2004). The removal of crayfish consumers from streams polluted with 2,4-D will cause periphytic algae, plankton and small invertebrates to rapidly increase in abundance, and completely shift the community structure of the ecosystem (Hanson et al. 1990; Charlebois and Lamberti 1996; Nyström et al. 1996; Keller et al. 1999). With crayfish no longer present to shred the detritus and organic matter, debris has the potential to build up and nutrient cycling will greatly decrease, diminishing the littoral productivity (Creed 1994; Charlebois and Lamberti 1996; Nyström et al. 1996; Phillips et al. 2009). In addition to a change in abundance of lower trophic level organisms, higher up trophic levels will also suffer due to the loss or impairment of crayfish exposed to 2,4-D (Creed 1994; Lodge et al. 1994; Dorn and Wojak 2004).

Aquatic food webs containing animals negatively impacted by 2,4-D are vulnerable to shifts in the community structure due to the impact of trophic cascades. These cascades can occur when a specific trophic level or organism has altered population abundance (Zale 1987). These changes can emerge from reduced foraging efficiency caused by sensory impairment in the target organism or decreased predation pressure which results in an increase in the abundance of prey. Trophic cascades can also occur when prey species experience population declines,
resulting in a reduction in growth and fitness of the predators that rely on the reduced prey populations (O’Connell and Raymond 1970; Houde and Schekter 1981, Brazner and Kline 1990). With lower level food sources eliminated, the higher trophic level consumers are forced to find other food sources, causing a shift in the community structure of the system (Ostfeld 1982; Anderson and Piatt 1999; Hansen et al. 2013). Studies exposing aquatic systems to pesticides have found trophic cascade effects in aquatic webs (Relyea and Diecks 2008). Increased amounts of the pesticide maltahion caused a decrease in the predation rates on amphibians without causing a decline in the number of predators, suggesting that pesticides can indirectly impact food webs through trait-mediated effects (Relyea and Hoverman 2008). Low concentrations of malathion were also shown to have indirect lethal effects on aquatic communities. These effects include a loss of zooplankton which initiated a trophic cascade, a bloom in phytoplankton, a decline in periphyton, and a reduction in growth and survival of the top predator, leopard frog (Relyea and Diecks 2008). In a similar behavioral assay with mummichog fish from clean and polluted environments, researchers found that predation rates of mummichogs taken from tributaries polluted by surrounding highways, industrial sites, and high levels of organic contaminants and metals were reduced (Weis et al. 2001).

Changes in food web structure can also occur through top-down effects in environments where predation pressures govern trophic structure (Frank et al. 2005). Top-down controlled communities could be very sensitive to changes in prey abundance in the effects of prey on their consumers, indicating that changes in the body mass or abundance of prey items will have significantly large effects on the consumers (Berg et al. 2011). The loss of predators in aquatic ecosystems can have deleterious top-down effects, causing an increase in the presence of plaktivorous fish, reducing the abundance of grazing zooplanktion and increasing the risk of
eutrophication due to the presence of excessive nutrients (Carpenter 2003; Frank et al. 2005). Due to the pesticide-induced mortality of predators, the presence of carbaryl pesticide caused indirect effects on community interactions and increased the survival of the tadpole prey (Mills and Semlitsch 2004). The introduction of newt predators shifted a cladoceran community previously dominated by a large *Daphnia* species to a community dominated by the smaller *Ceriodaphnia* and *Scapholeberris* species (Mills and Semlitsch 2004). Changes in abundance of aquatic species also occur when the impacted species exerts a bottom-up effect in aquatic communities.

Lake models have shown that the maximum attainable biomass for members of trophic levels is established by the effects of bottom-up energy processes (Mills and Schiavone1982; McQueen et al. 1986). Changes in abundance of a variety of higher level aquatic species occurred in studies that had bottom-up effects through an increase or decrease of food supply, or competitor release among consumers (Brazner and Kline 1990). Difubenzuron insecticide applied in mesocosms with zooplankton, phytoplankton and fish populations caused a direct reduction of the zooplankton and an indirect decrease in the survival and body mass of the bluegill predator fish due to their loss of prey items (Boyle et al. 1996). Herbicides have been shown to impact food webs through bottom-up effects by limiting food availability or by selectively killing primary producers (deNoyelles et al. 1982; Lampert et al. 1989; Jutner et al. 1995; Berard et al. 1999). Populations of central newts became emaciated and eventually died when the abundance of their cladoceran prey significantly decreased in the presence of carbaryl pesticide (Mills and Semlitsch 2004). Organisms that serve roles as both predators and prey can cause similar shifts in the aquatic community structure with the modification of their behavior, physiology or the reduction in their abundance. These findings are similar to the behavioral
modifications of crayfish during exposure to 2,4-D and are indicative of trophic cascades and shifts in food web structure that can occur when crayfish are behaviorally impaired by the herbicide.

Our results indicate that sub-lethal levels of 2,4-D causes significant behavioral and potential physiological changes in crayfish, *Orconectes rusticus*. Impacts were noted as significant in exposures as low as conditions mimicking LC10 levels of exposure, which are already present in streams and rivers in the United States (Smith and Isom 1967; U.S. EPA/OPP 2004). It is imperative that more rigorous efforts in monitoring and prevention be made by government organizations and professionals using the chemical at the largest scales. Contamination of 2,4-D in freshwater systems threatens to disrupt both behavioral and physiological processes of these essential species, potentially resulting in a cascade of deleterious effects through additional trophic levels. More studies need to be conducted to identify the behavioral impairments in aquatic species that result due to herbicide exposure below LC50 levels, especially when concerning a species of high importance in their ecosystem.
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**Figure 1:** Y-maze setup for foraging experiments. The maze is divided into three zones (A, B, C). Herbicide solutions and water are brought into the system from reservoirs through Nalgene® tubing. Flow of odor sources is constant through flowmeters. Solutions enters into arm A and B of the tank and is drawn out by five exit valves on the far right side of the maze. Figure has been drawn to scale.
Figure 2: Mean (± SEM) time to locate a food source within a Y-maze. Different shaded bars represent crayfish exposed to different levels of 2,4-D: no exposure control (open), low level of 2,4-D (solid gray), medium level of 2,4-D (hatched, light gray), and high level of 2,4-D (cross-hatched). N=19 for control and N=15 for each 2,4-D treatment group. Bars with different letters are significantly different from each other (One-way MANOVA Fisher’s LSD post-hoc, p<0.05).
Figure 3: Mean (±SEM) time in the correct arm of the maze. Different shaded bars represent crayfish exposed to different levels of 2,4-D: no exposure control (open), low level of 2,4-D (solid gray), medium level of 2,4-D (hatched, light gray), and high level of 2,4-D (cross-hatched). N=19 for control and N=15 for each 2,4-D treatment group. Bars with different letters are significantly different from each other (One-way MANOVA Fisher’s LSD post-hoc, p<0.05).
Figure 4: Mean (±SEM) walking speeds of crayfish during the presence of food odors. Different shaded bars represent crayfish exposed to different levels of 2,4-D: no exposure control (open), low level of 2,4-D (solid gray), medium level of 2,4-D (hatched, light gray), and high level of 2,4-D (cross-hatched). N=19 for control and N=15 for each 2,4-D treatment group. Bars with different letters are significantly different from each other (One-way MANOVA Fisher’s LSD post-hoc, p<0.05).
**Figure 5:** Mean (±SEM) overall speeds of crayfish during the presence of food odors. Different shaded bars represent crayfish exposed to different levels of 2,4-D: no exposure control (open), low level of 2,4-D (solid gray), medium level of 2,4-D (hatched, light gray), and high level of 2,4-D (cross-hatched). N=19 for control and N=15 for each 2,4-D treatment group. Bars with different letters are significantly different from each other (One-way MANOVA Fisher’s LSD post-hoc, p<0.05).
Figure 6: Mean (±SEM) percent consumption rates of fish gelatin food sources. Different shaded bars represent crayfish exposed to different levels of 2,4-D: no exposure control (open), low level of 2,4-D (solid gray), medium level of 2,4-D (hatched, light gray), and high level of 2,4-D (cross-hatched). N=19 for control and N=15 for each 2,4-D treatment group. Bars with different letters are significantly different from each other (One-way MANOVA Fisher’s LSD post-hoc, p<0.05).