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Effects of Nonylphenol on Orientation by the Crayfish *Orconectes propinquus*

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Dedication

This work is dedicated to my late grandfather, Robert H. Bennett, who always supported my sense of curiosity and fascination about the natural world. As a fellow devotee of nature, he encouraged my exploration of biology and continuously took an interest in my academic endeavors. Without his guidance and influence, I would not be here. Thank you for your legacy of learning and education.

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Abstract

Nonylphenol is a widely used surfactant subject to ethoxylation and subsists in the environment for significant amounts of time. It has industrial, agricultural, and domestic uses, and makes its way into the aquatic ecosystem despite treatment of wastewater. Detrimental effects of nonylphenol are varied, but most notably endocrine disruption has been examined. Crayfish are a crucial invertebrate in freshwater ecosystems. They are omnivorous, and occupy a key position in the trophic web as both predator and prey. To determine if nonylphenol exposure has any effect on the orientation abilities of crayfish, I acutely (1 day) and chronically (4 days) exposed crayfish to a sublethal amount of nonylphenol. The crayfish then attempted to locate a food odor in a modified Y-maze. Both acutely and chronically exposed crayfish were significantly less successful at choosing the food odor arm than controls, and acutely exposed crayfish also spent significantly less time in the food odor arm. These differences indicate sublethal nonylphenol exposure impairs the orientation ability of crayfish to a food source.

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BACKGROUND

Aquatic Pollutants

Human practices have created a great deal of various chemical pollutants. Industrial, agricultural, and household activities all use water on a daily basis, resulting in the release of numerous chemical compounds into the environment, including bodies of water. When water sources become polluted, the effects can be extensive, impacting not only wildlife, but human health as well. Even trace amounts of pollutants are of concern. Chronic exposure to low levels of pollutants may be even more alarming for aquatic organisms. Any consequences of exposure may be so subtle; they may escape detection for extended periods of time, until accumulation of these effects causes a larger, more significant impact (Daughton & Ternes, 1999).

There are two main categories for the sources of aquatic pollution. Point source pollution comes from "a discrete conveyance typically thought of as an effluent from the end of a pipe" (U.S. EPA, 2010). Examples of point source pollution are effluent from a wastewater treatment plant, and an outfall or discharge from a storm sewer. The other category is non-point source pollution, which is pollution that comes from a diffuse location and does not have a single, identifiable source (U.S. EPA, 2010). Examples include runoff, rainfall, and snowmelt.

Point Source pollution is much easier to control than Non-Point Source pollution through the Clean Water Act (CWA). The CWA was last amended in 1972 and set standards for the wastewater industry, surface water contaminants, and water quality. Under the CWA, it is illegal for industrial and municipal facilities to discharge any pollutants from a point source into surface waters unless the facility possesses a permit from the National Pollutant Discharge Elimination System (NPDES). Wastewater is treated via several processes before it leaves the treatment facility as effluent.

Wastewater Treatment

In an article by Huler (2010), the process of treating wastewater was described in several steps. First, the incoming wastewater passes through screens and cyclone filters to remove large and floating debris, which then go to a landfill. Next, the water flows into primary clarification tanks. At this point, the water flows very slowly, allowing particles to sink to the bottom and grease to float to the top. The surface is skimmed to remove the grease, which is added to the layer of sludge on the bottom via a small flume. The sludge is pumped out, and the water flows into large aeration tanks. Nozzles in the tank floor aerate the water. Aeration provides oxygen to the bacteria in the tanks, which feed on the waste and remove chemicals.

The aeration step is of particular interest. Water quality and cleanliness is indicated by a value known as the biochemical oxygen demand (BOD). The BOD is the amount of oxygen needed by the bacteria to process and remove organic chemicals, and is usually given as milligrams per liter (mg/L) (U.S. EPA, 1997). The higher the BOD, the more polluted the water. Accordingly, a lower BOD is desirable by wastewater treatment plants. Aeration is a costly step in the treatment process, as it uses energy to pump in the oxygen. If the BOD is lower, then aeration tanks can be smaller, resulting in less energy use (Huler, 2010). A more cost efficient way is using anaerobic digestion instead of aerobic digestion, which some wastewater treatment plants are using. With anaerobic digestion, no oxygen is needed, and energy is actually produced in the form of methane and carbon dioxide gas. These gases can be used to provide the treatment plant with useable energy, thus reducing costs and increasing efficiency (Coffey, 2009).

After aerating for about a day, the water then goes into the secondary clarification tanks. Again, the water flow is very slow, allowing the bacteria to sink and settle on the bottom. The sludge on the bottom is pumped out, and the water flows out near the top of the tanks. The

treatment process is nearly finished; with only sand filtering and UV light or chlorine treatment remaining. Sand filtering removes any remaining fine particles, and the UV light or chlorine inactivates pathogens. Then the water leaves the facility as effluent, and usually empties into a river or other body of water.

Household Pollutants

Despite being treated, many pollutants enter aquatic ecosystems in wastewater effluent (Ruhoy & Daughton, 2008; Santos et al., 2009). Effective treatment options for removing various pollutants from wastewater exist, but many come with drawbacks such as high maintenance costs and formation of harmful secondary pollutants in the process (Grassi et al., 2012). Sources of pollution are not limited to just agricultural and industrial products, but also include household products. Household pollutants can be pharmaceutical compounds, soap and cleaner ingredients, and even artificial sweeteners.

Many pharmaceuticals end up in aquatic ecosystems via wastewater treatment plant effluent, after being discarded directly and indirectly into the sewer system by the consumer (Ruhoy & Daughton, 2007; Ruhoy & Daughton, 2008; Santos et al., 2009; Brodin et al., 2013). Because they are used year-round, these compounds are continuously infused into the water. This results in a continuous exposure for aquatic animals, as opposed to fertilizers/pesticides, which are used in a more seasonal manner (Daughton & Ternes, 1999; Daughton, 2002).

Pharmaceutical compounds are of concern due to how they interact with biological organisms. They are designed to elicit effects at low concentrations and many can pass through membranes. Most compounds also are very polar and not very volatile, which increases their likelihood of ending up in aquatic ecosystems (Brausch et al., 2012). Some even have an affinity

for sediment, which may impact benthic habitats in aquatic ecosystems (Hagenbuch & Pinckney, 2012).

Types of pharmaceuticals found in wastewater effluent are varied. Anti-inflammatory drugs (ibuprofen, ketoprofen, naproxen), an anti-epileptic (carbamazepine), β-blockers (acebutolol, atenolol, metoprolol, propranolol, timolol, nadolol, labetalol, oxprenolol, pindolol, alprenolol), antibiotics (ciprofloxacin, macrolides, sulfonamides), anxiolytics (benzodiazepines), and of course, endocrine compounds (17-α ethinylestradiol/EE2, thyroid hormones) have all been detected in wastewater treatment plant effluent (Daughton & Ternes, 1999; Lee et al., 2007; Santos et al., 2009; Wise et al., 2011; Hagenbuch & Pinckney, 2012; Martinez et al., 2012; Salem et al., 2012; Brodin et al., 2013; Samaras et al., 2013). While wastewater treatment plants remove a percentage of these compounds from sewer system influent, the fact that so many remain at detectable levels after treatment is concerning. The amounts of these pollutants remaining in treated effluent have the potential to affect aquatic organisms, especially in immediate areas of wastewater effluent discharge. For example, the antibiotics tylosin and lincomycin affected a lower trophic level by reducing motility in two species of marine diatoms (Hagenbuch & Pinckney, 2012). The anti-anxiety drug oxazepam elicited negative effects at a higher trophic level by increasing activity, feeding rate, and reducing socialization in European perch (*Perca fluviatilis*) (Brodin et al., 2013). Thus, even though levels of pharmaceuticals in wastewater effluents are usually very low, researchers are still concerned.

Triclosan is an antimicrobial compound found in many everyday household products, including liquid hand soaps, hand sanitizers, facial cleansers, and even toothpaste. It is also an ingredient in Microban, an antibacterial additive used in plastics. Microban has been incorporated in a wide variety of household products, such as hairbrushes, plastic cutting boards,

and shoe inserts. Due to its hydrophobic nature, up to 90% of triclosan can be removed during wastewater treatment, but despite this, it still remains at detectable levels in effluent (Buth et al., 2010).

The primary concern with triclosan in the environment is its transformation by chlorine and sunlight. In the last step of wastewater and drinking water treatment, chlorine is often used to disinfect the water before it leaves the facility. The free chlorine can react with the triclosan, forming three new compounds, called chlorinated triclosan derivatives (CTDs). These CTDs plus the excess triclosan then leave the treatment plant as effluent. In the aquatic environment, the CTDs and triclosan can be further transformed by sunlight. Upon exposure to sunlight, the CTDs and triclosan undergo a photochemical reaction, which results in four polychlorodibenzo-*p*-dioxins (PCDDs) (Buth et al., 2010; Anger et al., 2013; Williams, 2013). PCDDs are known human carcinogens and can also have detrimental effects on cardiovascular, liver, and endocrine function (Bertazzi et al., 2001; Weir, 2005; Marinkovic et al., 2010).

Artificial sweeteners are used in a wide variety of foods, beverages, drugs, and even animal feed (Buerge et al., 2011; Lange et al., 2012). They are very stable compounds, also very water soluble, and very little is absorbed in human digestion, yielding up to 90% of consumed amounts in human excretions (Wiklund et al., 2012; Tollefsen et al., 2012). Because they are not easily metabolized or absorbed, artificial sweeteners are not a viable energy source, hence their ubiquitous use in dietary products (Tollefsen et al., 2012). Their stability and water solubility makes their removal in treatment plants difficult, resulting in removal efficiencies of only 32-48% under the best conditions (Soh et al., 2011). Tollefsen et al.'s review (2012) also found that concentrations of artificial sweeteners in effluent were most often found to be in the range of 0.4-11 µg/L.

Research conducted by Huggett & Stoddard (2011) found no significant effects of sucralose exposure on two crustaceans, *Daphnia* and mysid shrimp. Conversely, Wiklund et al. (2012) found sucralose to impact behavior in both *Daphnia* and amphipods. After sucralose exposure, *Daphnia* displayed increased swimming speed and altered swimming height, while the amphipods took significantly longer to locate food and shelter. Soh et al. (2011) found the aquatic plant *Lemna gibba* exhibited no adverse effects of sucralose exposure, but also highlighted the stability of sucralose and its persistence in the aquatic environment, possibly leading to low-dose, chronic exposure to aquatic organisms.

Chemical Risk Assessment

The U.S. Environmental Protection Agency (EPA) is the part of the federal government responsible for regulating pesticides and other chemicals, ensuring they "do not pose unreasonable risks to human health or the environment" (U.S. EPA, 2007). The EPA sets regulations regarding use and exposure based on assessments of these chemicals. For evaluation and approval, chemicals go through a process called Risk Assessment, which consists of four steps.

The first step is hazard identification. Potential effects on human health from chemical exposure are determined and reviewed. Data on these effects may be compiled from existing sources, or tests may be conducted to generate new data. Toxicology tests are typically run on vertebrate animals in laboratories, and evaluate length of exposure (acute, intermediate, chronic), type of exposure (oral, dermal, inhalation), and carcinogenicity. Also observed are reproductive and developmental effects, as well as endocrine disruption (U.S. EPA, 2007).

Step two is dose-response assessment. The EPA must also assess how much of a chemical a person may be exposed to before harmful effects occur. Some chemicals may not be harmful at small exposures, but may elicit harmful effects at higher exposures. These dosages are determined from the observed effects on test animals, and calculated to a scaled dose for humans (U.S. EPA, 2007).

Exposure assessment is the third step. This entails estimating the exposure level a person will encounter. Exposure can occur by several means. Chemicals can be ingested when present on food, such as produce or in drinking water. Exposure can also occur via use of home products such as cleaners, pesticides, insect repellants, and flea/tick prevention for pets. People who work in jobs where chemical use is common (agriculture, pest control, etc.) may be subject to occupational exposure. The level of exposure is estimated based on documents and reports, which take into account the chemical's source, usage patterns, production processes, and environmental fate (U.S. EPA, 2007).

The fourth step is risk characterization. Data from the previous steps are combined to report the overall risk of using the chemical. The overall risk to human health of using a chemical is dependant on both its toxicity and predicted exposure. Uncertainty factors are added to the risk assessment, which may be up to 10-fold and create a wider margin of safety (U.S. EPA, 2007). After the Risk Assessment is completed, the information is then used in the decision to approve the use of a chemical or pesticide.

The EPA's Risk Assessment process is not without its criticisms. One of the main problems with the process is the lengthy amount of time it takes to complete an assessment (Trager, 2009; Gray & Cohen, 2012; Trager, 2012). Assessments taking years to complete delay the release of information and take up valuable time and resources in evaluating newer

chemicals. Some chemical assessments get pulled into cycles of revisions, despite already being evaluated. Due to this, many chemicals have never been evaluated (Gray & Cohen, 2012; Trager, 2012). The EPA's Integrated Risk Information System (IRIS) is a public database, which contains the completed risk assessments. Currently, the database houses 557 assessments of the roughly 80,000 chemicals in use (Trager, 2009; Gray & Cohen, 2012). Quicker, "rough and ready" estimates would speed up the process and be sufficient enough for policy-making (Gray & Cohen, 2012; Trager, 2012). It is worth noting that risk assessments for nonylphenol and octophenol are not currently in the IRIS database.

Another criticism involves the methods of collecting and reporting the assessment data. When collecting data on low levels of exposure using animal models, the current system assumes any harmful health effects observed in the animals can be reproduced in humans. It also assumes the high exposures administered to animal models can be calculated downward to yield exposure levels appropriate for human populations (Gray & Cohen, 2012). In an effort to protect human health, the EPA often overestimates risk when the available data is insufficient (Gray & Cohen, 2012, Trager 2012). As a result, the assessment may be misleading to policymakers, leading to inaccurate, overly rigid regulations, and ultimately resulting in "sub-optimal protection of public health" (Gray & Cohen, 2012, Trager 2012).

Conclusion

There are many sources of aquatic pollution, from large-scale industrial sources to smaller, individual household sources. The U.S. EPA has been charged with evaluating chemicals and creating regulations regarding their use via their risk assessment procedures. While put in place to ensure public health and safety, this system is not always efficient, and

many chemicals have not yet been evaluated. Complete data regarding newer chemicals is often lacking, yet use of these chemicals continues. The wastewater treatment process is effective at reducing the concentration of pollutants in water, but many still remain in effluent at detectable levels.

Effects of these remnant pollutants can have widespread impacts in the aquatic environment. Some pollutants (including nonylphenol) are hydrophobic and will settle out of the water column and adhere to the sediment, while others are hydrophilic and persist in the water column. Hence, all aquatic organisms are exposed to aquatic pollution, from the benthic (crayfish, snails, worms) to the pelagic (fish, water insects).

With the multitude of chemicals in use, and the potential for non-target species to be affected, considerable research must continue. By examining the effects of chemicals found in the aquatic environment, we gain insight on their impacts on common aquatic inhabitants, which may alter entire food webs. In time, this may lead to updated regulations regarding the careful use and labeling of potential pollutants, including nonylphenol.

EXPERIMENTAL

Introduction

Nonylphenol

Nonylphenol is an alkylphenol used as a surfactant in a wide variety of industrial, household, and agricultural settings. Industrially, it is used in paints, plastics processing, paper mills, detergents, and lubricants. Domestic use includes detergents and household cleaners. Agriculturally, it is found in pesticides and herbicides. Nonylphenol has both hydrophobic and hydrophilic components (amphiphilic), enabling it to reduce surface tension between two liquids or a liquid and a solid. The ability to break up oil in water, allow other chemicals to adhere to plant leaves, and allow mixing between polar and nonpolar compounds makes it a common ingredient in these products.

Currently, nonylphenol is listed as an "inert ingredient" contained within pesticides and herbicides. Nonylphenol can be found on the U.S. Environmental Protection Agency's List of Inert Pesticide Ingredients 4B, which is compiled of "other ingredients for which EPA has concluded they have sufficient information to reasonably conclude that the current use pattern in pesticide products will not adversely affect public health or the environment" (U.S. EPA, 2004). Inert ingredients can be approved by the FDA by submitting a petition to the EPA's Inert Ingredient Assessment Branch (IIAB) along with supporting documents and data. The application and supporting information are reviewed by the IIAB and a decision is made regarding the safety of the proposed ingredient (U.S. EPA, 2013).

Regarding freshwater ecosystems, the EPA has designated two types of exposure criteria. The first is acute exposure, which is the average concentration of nonylphenol over a 1-day period, and does not exceed 28 μg/L more than once every 3 years on average. The second is

known as chronic exposure and is the average concentration of nonylphenol over a 4-day period and does not exceed 6.6 μg/L more than once every 3 years on the average. Outside of the United States, nonylphenol and nonyphenol ethoxylates have been restricted by the European Union as a hazard to human and environmental safety.

Nonylphenol is a nonpolar compound with a high octanol-water partition coefficient (K_{ow}, 4.48), allowing it to easily bind to soil particles (Ferrara et al., 2001). Alkylphenols often settle in areas of low oxygen, including rivers, streams, and sediments where crayfish are predominantly found (Ying et al., 2002; Soares, 2008). Studies of the actual time nonylphenol remains in sediment yields mixed results. A review by Ying et al. (2002) references several studies, including Ekelund et al. (1993) that found the half-life to be 58 days in aerobic marine water and Marcomini et al (1989) that found nonylphenol ethoxylates to rapidly degrade initially, yet still yield residual concentrations detectable after 320 days. Shang et al. (1999) estimated the half-life of the ethoxylate groups to be as high as 60 years based on examination of sediment cores showing no signs of degradation with depth.

Nonylphenol occurs as a breakdown product of alkylphenol ethoxylates, specifically nonylphenol ethoxylates. These alkylphenol ethoxylates reach the environment largely by wastewater treatment plants (Hong and Li, 2007), where initial breakdown of the ethoxylate groups begins (Ying et. al., 2002). With alkylphenol ethoxylates, the ethoxylate groups can be easily lost, leaving the alkylphenol group intact (Figure 1). A study by Gonzalez et al. (2010) analyzed sewage sludge from 20 wastewater treatment plants for nonylphenol and nonylphenol ethoxylates. Levels of nonylphenol were frequently found in excess of the 50 mg/kg limit set by the European Union, with a mean concentration of 88 mg/kg dry matter (Gonzalez et al., 2010). When drain water was analyzed from an agricultural region, the concentration of

nonylphenol ranged from 0.5 µg/L to 6.0 µg/L (Zgoła-Grzeskowiak et al., 2009). Hernandez-Raquet et al. (2007) focused on treatment to remove nonylphenol from sewage sludge and found aerobic treatment yielded the best results, with near 100% removal rates of nonylphenol. Contrary to expectations, they also found anaerobic treatment resulted in higher concentrations of nonylphenol than before treatment (Hernandez-Raquet et al., 2007). Crayfish are benthic organisms, occupying the bottom of streams, walking along the sediment, and living among structures such as rocks and logs. Sediment is a more anaerobic environment, and nonylphenol is likely to settle there, resulting in higher concentrations than in the water column.

Nonylphenol is also a known endocrine disruptor. Its chemical structure is similar to that of estrogen, allowing it to interact with estrogen receptors (Figure 2). Many studies have focused on these endocrine disruption effects in an aquatic context. For example, notable feminization effects occur in fish, such as an increase in vitellogenin (egg yolk protein), which occurred in a dose-dependent manner in male rainbow trout (*Oncorhynchus mykiss*) (Jobling & Sumpter, 1993; Schwaiger et al. 2002). Additionally, the offspring of the nonylphenol-exposed fish showed increased estradiol levels in males and increased testosterone levels in females (Schwaiger et al., 2002). Evidently, nonylphenol's endocrine disrupting ability is already affecting fish, including their offspring. The ability to affect subsequent generations of a fish population could have major ecological impacts over time.

The effects of nonylphenol and other endocrine disruptors on invertebrates have been well documented (review by Depledge et al., 1999, review by Rodriguez et al., 2007). Marine copepods exhibited high mortality when exposed to nonylphenol at a concentration of 62 µg/L, while a concentration of 125 µg/L was acutely lethal to juveniles (Bechmann, 1999). Significant reductions in growth resulted when marine mysid shrimp were exposed to sublethal

concentrations of nonylphenol, along with a decrease in the number of molts (Hirano et al., 2009). A study by Lye et al. (2008) found that male shore crabs showed a reduction in testis weight, an increase in liver weight, and changes in ecdysteroid levels. Freshwater amphipods collected from a heavily polluted bay were found to have lower sperm counts and a higher proportion of intersex males (Yang et al., 2008).

Evidence of endocrine-disrupting effects from nonylphenol in mammals also has been found. Nonylphenol exposure increased mitotic activity in rat endometrium, and there is also some evidence showing nonylphenol inhibits rat stem cell growth (Soto et al., 1991; Kudo et al., 2004). Nonylphenol exposure reduced or eradicated rat sperm motility, and the smallest concentration (1 μg/ml) was enough to damage the acrosome, while higher concentrations induced the acrosomal reaction (Uguz et al., 2009).

Human implications from nonylphenol already have been documented. One study found nonylphenol bound to estrogen receptors and stimulated cell proliferation in human breast tumor cells (Soto et al., 1991). Evidence of prenatal exposure to nonylphenol has been detected, with higher levels found in urban areas (Chen et al., 2008). Additionally, a correlation between higher nonylphenol exposure and a younger age of menstruation was observed in girls going through puberty (Chen et al., 2009). Lagos-Cabre & Moreno (2012) discuss the involvement of nonylphenol and other endocrine-disrupting chemicals on male infertility, suggesting they interfere with regulated apoptosis during spermatogenesis.

Although the existing research mainly focuses on the endocrine disrupting effects of nonylphenol, a smaller number of studies have assessed behavioral changes. After nonylphenol exposure, both male and female zebrafish (*Danio rerio*) exhibited a decrease regarding group preference, preferring to isolate themselves from the safety of the school.

Females also showed an increase in aggression (Xia et al., 2010). Ward et al. (2006) found that after a 5-day exposure to nonylphenol, juvenile rainbow trout females were less successful at foraging, reduced their shoaling tendency, and thus were more likely to be attacked by other fish.

Crayfish

Crayfish are a crucial invertebrate in many rivers, streams, and other freshwater ecosystems. Because of the positions they occupy in the trophic web, crayfish can have a major impact on their habitat and surrounding areas (Steele et al., 1992; Usio & Townsend, 2002), making them a sentinel species in many aquatic habitats (Anton et al., 2000). Depending on the availability of resources and their life stage, crayfish can play a functional role in up to three different trophic levels (VanArman, 2011). Some crayfish species remain flexible in their trophic position, with an ability to adapt to available resources, while some crayfish species stay in a more fixed position, with little alterations in diet (Johnston et al., 2011). By consuming both smaller aquatic organisms and organic matter, they are omnivorous and serve an essential role in the decomposition pathway. A study by Usio & Townsend (2002) found the presence of crayfish increased leaf decomposition and decreased predatory invertebrate densities in a freshwater stream habitat. When gut contents and diet were analyzed in *Orconectes propinquus* and *O. rusticus* crayfish, it was found that as much as 42% of their diet was carnivorous food, with 12% of the total diet being fish matter, suggesting crayfish may have a bigger impact on fish populations than previously thought (Taylor & Soucek, 2010). In addition, crayfish serve as an important food source for animals in higher trophic levels (including humans). In the some ecosystems, crayfish serve as the primary food

source for more than 40 kinds of vertebrates (VanArman, 2011). Hence, all animals and humans may be exposed to nonylphenol when exposed crayfish are consumed as a food source.

In their aquatic environment, crayfish and other decapod crustaceans are surrounded by different odors and chemical signals. They have two main pairs of sensory appendages: the longer and larger touch antennae, and the shorter and smaller smell antennules.

The antennules (first antennae) of the crayfish are the primary olfactory organs. The antennules are spatially separated, consisting of a lateral and a medial ramus (Figure 3). Hair-like structures, called setae, are distributed over the antennules, and have been studied in several decapod crustaceans. In six species of the *Lysmata* shrimp, researchers identified four different types of setae (Zhu et al., 2011), while researchers studying the Caribbean spiny lobster *Panulirus argus* identified ten different setal types (Cate & Derby, 2001). Hallberg et al (1997) looked at setae across several crustaceans, including the decapods *Pacifastacus leniusculus* (signal crayfish), *Carcinus maenas* (common shore crab), *Crangon crangon* (brown shrimp), and *Hyas arnneus* (great spider crab), and found the arrangements and density of setae varied between animals. Setae also can be found on the walking legs and chelae in crustaceans (Grasso & Basil, 2002; Belanger & Moore, 2006). Setae can be either chemosensory for responding to chemical cues, mechanosensory for responding to water flow, or both (bimodal). Many studies have been conducted regarding aesthetascs, a key type of setae. Aesthetascs possess chemosensory abilities and are located on the distal area of the lateral rami (Tierney et al., 1986). They are innervated by olfactory receptor neurons, which extend to the ipsilateral olfactory lobe in the brain (Sandeman & Luff, 1973; Mellon, 2012). The antennules are constantly acquiring olfactory information about their surroundings via chemical cues. These cues are critical to their survival and are used in several behaviors

related to foraging (Moore & Grills, 1999; Grasso & Basil, 2002), determining social information and status (Atema, 1995; Bergman et al., 2003; Johnson & Atema, 2008), and mate odors (Giri & Dunham, 2000; Aquiloni & Gherardi, 2008).

Crayfish orientation to an odor source has been explored through two kinds of taxis, chemotaxis and rheotaxis. Chemotaxis is when an organism moves towards (positive) or away from (negative) a chemical stimulus by moving up or down a chemical's concentration gradient. Rheotaxis is when an organism orients itself to face towards (positive) or away from (negative) an oncoming water current. Mechanoreceptors on crayfish antennae detect changes in water flow. Crayfish will engage in positive rheotaxis in response to these changes, and will also turn and face small currents produced by mobile prey (Ebina & Wiese, 1984; Breithaupt et al., 1995). They show a positional preference when interacting with conspecifics in a flow environment (Bergman et al., 2006). Research has found that the type of substrate may play a role in the efficiency of orientation when crayfish engage in a chemically mediated rheotaxis (Moore & Grills, 1999).

Ablation studies (removal of antennules) have shown mixed effects on chemotaxis. American lobsters (*Homarus americanus*) were not able to locate a food source at a distance when their right antennules were ablated (Beglane et al., 1997). Similarly, rusty crayfish (*Orconectes rusticus*) were significantly less successful than intact crayfish at locating a food source with left, right, or both antennules lesioned (Kraus-Epley & Moore, 2002). These results suggest both antennules are needed for spatial information when orienting to a stimulus. However, Dunham et al. (1997) found that both inner & outer rami together were not necessarily needed to detect a sugar solution in *Cambarus bartonii*, but rather each kind of rami was sufficient on its own. Caribbean spiny lobsters (*Panulirus argus*) successfully

located a food source using either aesthetasc sensilla or non-aesthetasc sensilla, suggesting each of these structures alone is sufficient for this behavior (Horner et al., 2004).

Because their antennules are continuously being exposed to different chemicals, any pollutants in the water could have an adverse effect on olfactory-dependent behaviors (Steele et al., 1992). Pollutants could alter crayfish behavior by masking odors, inhibiting detection by chemoreceptors or changing the behavioral response to a chemical signal. By observing how nonylphenol affects olfactory-mediated behaviors, I can gain insight into the extent of the effects of sublethal exposure of nonylphenol on an aquatic invertebrate. Since crayfish depend on olfaction for a wide variety of behaviors, I examined if nonylphenol had any effect on the orientation ability of crayfish. I hypothesized that crayfish exposed to nonylphenol would be less successful in locating a food odor source than crayfish not exposed to nonylphenol.

Methods

Exposure setup

A total of 120 adult intact-intermolt male and female crayfish *Orconectes propinquus* were used for this study (60 male; 60 female). The crayfish were collected by seine net from tributaries of the Grand River. The crayfish were each kept individually in a small tank with 1L of water and an air bubbler (Figure 4). All crayfish were kept in isolation for five days prior to experimentation to remove any influence of social cues and status ranking during the experimental trials.

For the exposure groups, a concentration of 0.05 μL of nonylphenol (1 ppm, sublethal concentration) was added to 1L of water in the isolation tank. This concentration was used as previous work in the lab had shown higher concentrations of nonylphenol to be acutely lethal

to crayfish. I dissolved 5.0 µL of nonylphenol into 10 mL of acetone, and then added 1 mL of this solution to the isolation box. Crayfish for the acute exposure trials (n = 30, 15 males and 15 females) were kept individually in isolation tanks with 1L of the nonylphenol water for a duration of 24 hours. Crayfish for the chronic exposure trials (n = 30, 15 males and 15 females) were kept individually in isolation tanks with the nonylphenol water continuously for four days.

For the control groups, a vehicle solution of 1mL acetone was added to the water in the isolation tank. Crayfish for the acute and chronic control groups (n = 60, 30 males and 30 females) were also kept individually in isolation tanks with 1L of water and 1 mL of acetone for 24 hours and 4 days, respectively. All crayfish were kept on a 12:12 light-dark cycle in the laboratory, and were not fed over the duration of the experiment. This was done to enhance the response for the food odor in the behavioral assay.

Stimulus

The odor of food was used as the motivation in the behavioral assay. The food odor was obtained by pureeing a fillet of frozen perch in water, then pouring the puree over a fine mesh sieve and collecting the puree water. This water was then used to fill two 60-mL syringes, one containing 60 mL and one containing 40 mL for a total of 100 mL of food odor water for each trial.

Tank

Similar to the design used by Adams et al. (2003), a modified Y-maze tank was used for these trials. The tank was rectangular in shape, was constructed from black acrylic, and

had overall dimensions of 152 cm long, 72 cm wide, and 15 cm deep. It contained three areas: left arm, right arm, and end section. The arm divider was 100 cm long, which gave each arm a width of 36 cm (Figure 5).

Tap water flowed into the two arms (inflow arms) through a piece of 1.27 cm diameter clear plastic tubing 237 cm long. One end was attached to a tap water source, and the other attached to a plastic T-connector. The two remaining ends of the T-connecter each had a 9.0 cm long piece of tubing connecting it to another T-connecter serving as the odor injection port for each arm. The injection port was then connected to the inflow holes in the tank by a piece of tubing 30 cm long. The tank had two inflow holes of 0.64 cm diameter drilled into the end of the tank, one serving each arm. The remaining end of the odor injection port had a 3 cm long piece of tubing attached, which was plugged with a rubber stopper between food odor injections.

The outflow was at the opposite end of the tank, in the end section. Water flowed out of the tank through seven small outflow holes evenly spaced at the end of the tank, each with a diameter of 3/16 in. To help drain the tank quickly after each trial, three larger holes each with a diameter of 0.5 in. were drilled into the outflow end of the tank. These three holes were plugged with rubber stoppers during each trial, and the stoppers removed at the end of each trial. The tank was drained and then rinsed three times before each trial. Dye trials were done to evaluate flow through the tank.

Behavioral Assay

The purpose of the behavioral assay was to examine the crayfish olfactory ability after exposure to nonylphenol. All crayfish were subject to the behavioral assay. For each trial, the

tank was filled to a depth of 10 cm with water, and a crayfish was selected and placed into the end section of the tank promptly after exposure. The crayfish was placed beneath a wire cage to allow it to acclimate to the tank water for 5 minutes (Figure 6). At the end of this 5-minute period, the basket was removed and the crayfish was allowed to explore the tank. The crayfish were given a 10-minute trial where 20 mL of water with food odor present was injected at the start of the trial into the inflow of one of the randomly selected arms (Target) (Aquiloni & Gherardi, 2008) via the 60 mL syringe inserted into a port attached to the inflow tubing. The other inflow arm (Non-Target) received water only. An additional burst of 20 mL of food odor was injected at every 2-minute interval until the end of the trial (2, 4, 6, and 8 minutes). At the end of the trial, the crayfish was returned to a population tank and monitored for mortality (Figure 4).

Analysis

During this trial, the position and orientation of the crayfish was observed, and the time was recorded when it moved into a different section of the tank. The total time spent in each section and initial arm choice was recorded, along with the success of orienting towards the target arm. Instances for successful arm choice were analyzed using a multiple comparisons for proportions contingency table ($q_{0.05,\infty,3} > 3.314$) that allows for testing analogous to the Tukey or Student-Newman-Keuls tests (Zar, 1999). Significant results are represented by giving a $q_{0.05,\infty;3} > 3.314$ from the multiple comparisons test ($p < 0.05$). The multiple comparisons test was set up using a Microsoft Excel spreadsheet. Total time spent in each section was compared using a 2-tailed *t*-test, with significance reported at $p < 0.05$.

Results

Initial Arm Choice

Exposure to nonylphenol had an effect on orientation behavior in crayfish. Crayfish that were acutely exposed to nonylphenol were significantly less successful at initially choosing arm with the food odor stimulus than control crayfish, with a proportion of 0.500 choosing the target arm versus a proportion of 0.759 control crayfish ($q = 5.74$; $p < 0.05$ Fig. 7A). Crayfish from the chronic exposure group were also significantly less successful at initially choosing the target arm than the control group, with a proportion of 0.533 choosing the target arm, as opposed to the proportion of 0.759 control subjects ($q = 4.15$; $p < 0.05$ Fig. 7B). When crayfish from the two exposure groups were compared to each other, no significant difference was found regarding a successful choice of the target arm ($q = 1.50$; $p > 0.05$; Fig. 7C).

There was no side bias (left or right) exhibited by either the exposed or control crayfish. No significant difference was found between the acutely exposed and control crayfish choosing the right or left arm ($q = 2.15$; $p > 0.05$; Fig. 8A). Additionally, no significant difference was found between the chronic exposure and control groups choosing the right or left arm ($q = 0.474$; $p > 0.05$; Fig. 8B).

Time Analysis

Nonylphenol exposure also had an effect on the total time spent in the target arm. Acutely exposed crayfish spent nearly the same amount of time in each arm with 189 ± 35 (mean ± SEM) seconds spent in the target arm and 171 ± 32 seconds spent in the non-target arm, indicating no significant difference in the time spent in either the target or non-target arms ($p = 0.71$). Crayfish from the acute exposure group spent significantly less time in the target

arm than control crayfish (302 ± 32 sec; $p = 0.02$; Figure 9). Chronically exposed crayfish spent 210 ± 34 seconds in the target arm and 184 ± 34 seconds in the non-target arm, indicating no significant difference in the time spent in either the target or non-target arms ($p = 0.58$). There was a significant difference in time spent in the target arm between chronically exposed crayfish compared to controls ($p = 0.05$; Figure 9). Additionally, there was no significant difference found in the time spent in the target arm between the acute and chronic exposure groups ($p = 0.65$; Figure 9). Control crayfish spent a highly significant amount of time in the target arm than the non-target arm, with 302 ± 32 seconds in the target arm and 95 ± 21 seconds in the non-target arm ($p < 0.001$).

Discussion

The results show that nonylphenol exposure has a significant effect on the orientation ability of crayfish concerning locating a food source. Interestingly, just 24 hours of exposure to trace levels of nonylphenol was sufficient to significantly impact the ability of the crayfish to locate a food source. Chronically exposed crayfish experienced similar ramifications as acutely exposed crayfish, as they were also significantly impaired at locating the food odor. This agrees with our hypothesis that nonylphenol-exposed crayfish would be less successful at locating a food stimulus.

The length of nonylphenol exposure, however, did not seem to have a strong effect on crayfish behavior because there was no significant difference in success of choosing the target arm between the acute- and chronic-exposure groups. This suggests that impairment is brought about by very limited exposure to nonylphenol. Both groups of acutely and chronically exposed crayfish were significantly less successful at initially choosing the target arm than

controls, which indicates some kind of impairment, possibly involving the sensory or motor systems.

Exposure to nonylphenol also impacted the attraction time of crayfish to the target arm. Acutely and chronically exposed crayfish spent significantly less time in the target arm than control crayfish, which again agrees with my hypothesis. Exposed crayfish exhibited no significant difference between the time spent in the target and non-target arm. Not only were they less successful at initially choosing the target arm, but they were also not enticed enough by the food odor to stay in that vicinity. Clearly, nonylphenol exposure is having a disruptive effect on the olfactory abilities and orientation of crayfish to food odor.

The mechanisms and physiological explanations of these olfactory effects have yet to be determined. Nonylphenol exposure could affect the setae on the antennules in several different ways. The nonylphenol could damage the setae morphologically by altering its size, shape, and/or abundance. It might also be physiologically damaging by inhibiting olfactory-receptor neurons or affecting action potentials from both sensory and motor neurons. Because nonylphenol is hydrophobic, it has a tendency to settle into sediment and onto structures such as rocks, logs, etc. It is also possible that the nonylphenol could be adhering to the antennules and chemoreceptors, blocking the reception of some or all of a chemical signal. Moreover, nonylphenol is very viscous and may remove odors from the water by dragging them to the sediment. Further research is needed to identify the manner in which nonylphenol interferes with a crayfish's ability to find food.

I demonstrated that trace amounts of nonylphenol significantly impact the ability of crayfish to locate a food odor. While my chronic exposure was 4 days, the effects of longer-term exposure have yet to be determined. Olfactory impairment was a clear alteration, but

other more subtle effects may have gone undetected. Daughton & Ternes (1999) pointed out that chronic exposure to trace amounts of pollutants might cause imperceptible changes. These changes may be so small that they go unnoticed for years until they cumulate in a larger, more obvious ramification.

Future experiments that should be particularly useful include assessing nonylphenol in a dose-dependent manner and altering the duration of exposure. A close examination of the setae using a scanning electron microscope or similar technique after exposure also is warranted. It would be interesting to see if crayfish can recover from the effects of nonylphenol exposure, and whether molting repairs any damage to the antennules. Studies measuring the output and strength of olfactory-receptor neurons would provide some insight into physiological effects of exposure as well.

While the concentration of nonylphenol used in this experiment was not lethal, it was not without its detrimental effects. Only 24 hours of exposure were needed to impair the olfactory and orientation abilities of the crayfish. If crayfish experience an olfactory impairment, then long-term impacts of nonylphenol toxicity could be far-reaching, affecting their ability to find food as well as other olfactory behaviors. The means by which crayfish select a mate, avoid predators, and avoid contaminants in their habitat are all olfactory-dependent behaviors. If crayfish are unable to successfully navigate these tasks, then the consequences could alter entire ecosystems. With such an essential position within the trophic web, it is crucial to ensure the survival of the crayfish and its role in the aquatic ecosystem.

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Figure Legends

Figure 1. Degradation pathway of alkyphenol ethoxylates to an end product of the remaining alkylphenol. (From Ying et al., 2002)

Figure 2. Estrogen (left) and nonylphenol (right). The similarity in structures enables nonylphenol to interact with estrogen receptors. (From Soares et al., 2008)

Figure 3. Crayfish anterior view where the top arrow indicates a right lateral ramus, and the bottom arrow indicates a right medial ramus.

Figure 4. Individual isolation boxes where crayfish were kept 5 days prior and during experimentation (top), and population tanks where crayfish were kept after experimentation (bottom).

Figure 5. Y-maze tank. The tank featured two arms (left and right) and an end section. Inflow is on the right end and outflow is on the left end.

Figure 6. Crayfish isolation prior to the behavioral assay trial. Each crayfish was placed in the center of the end section with a wire basket over it to keep it in place during the 5-minute acclimation time.

Figure 7. Proportion of successful initial choice of the target arm. Asterisk indicates significance between experimental and controls. A) Acutely exposed crayfish were significantly less successful at choosing the target arm than controls ($q = 5.74$; $p < 0.05$). B) Crayfish from the chronic exposures were also significantly less successful than controls ($q = 4.15$; $p < 0.05$). C) There was no significant difference in success between the acute and chronic exposures ($q = 1.50$; $p > 0.05$).

Figure 8. Arm choice side bias. A) Acutely exposed crayfish did not choose the right arm significantly more or less than control crayfish ($q = 2.15$; $p > 0.05$). B) Chronically exposed crayfish did not choose the right arm significantly more or less than control crayfish ($q = 0.474$; $p > 0.05$).

Figure 9. Total time spent in the target and non-target arms for each group. Different letters indicate significant differences between experimentals and controls.

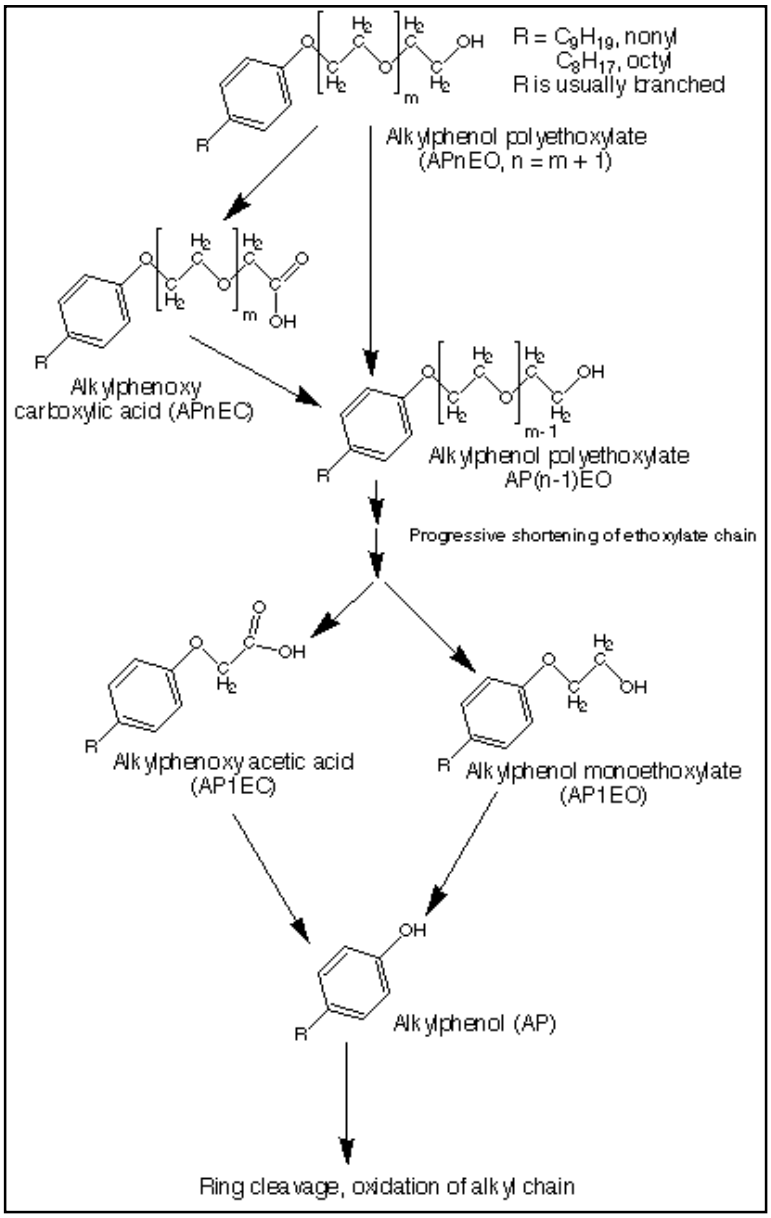

R = C_9H_{19}, nonyl
C_8H_{17}, octyl
R is usually branched
Alkylphenol polyethoxylate
(APnEO, n = m + 1)
Alkylphenoxy
carboxylic acid (APnEC)
Alkylphenol polyethoxylate
AP(n-1)EO
Progressive shortening of ethoxylate chain
Alkylphenoxy acetic acid
(AP1EC)
Alkylphenol monoethoxylate
(AP1EO)
Alkylphenol (AP)
Ring cleavage, oxidation of alkyl chain

Figure 1.

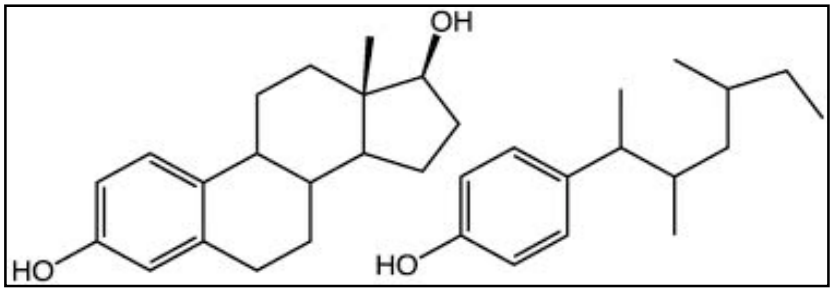


Figure 2.

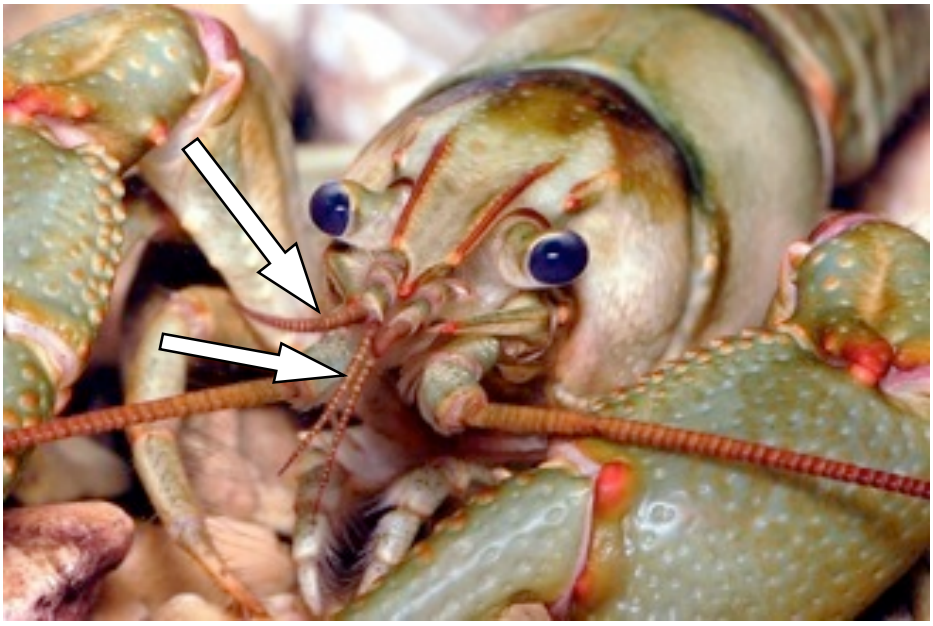


Figure 3.

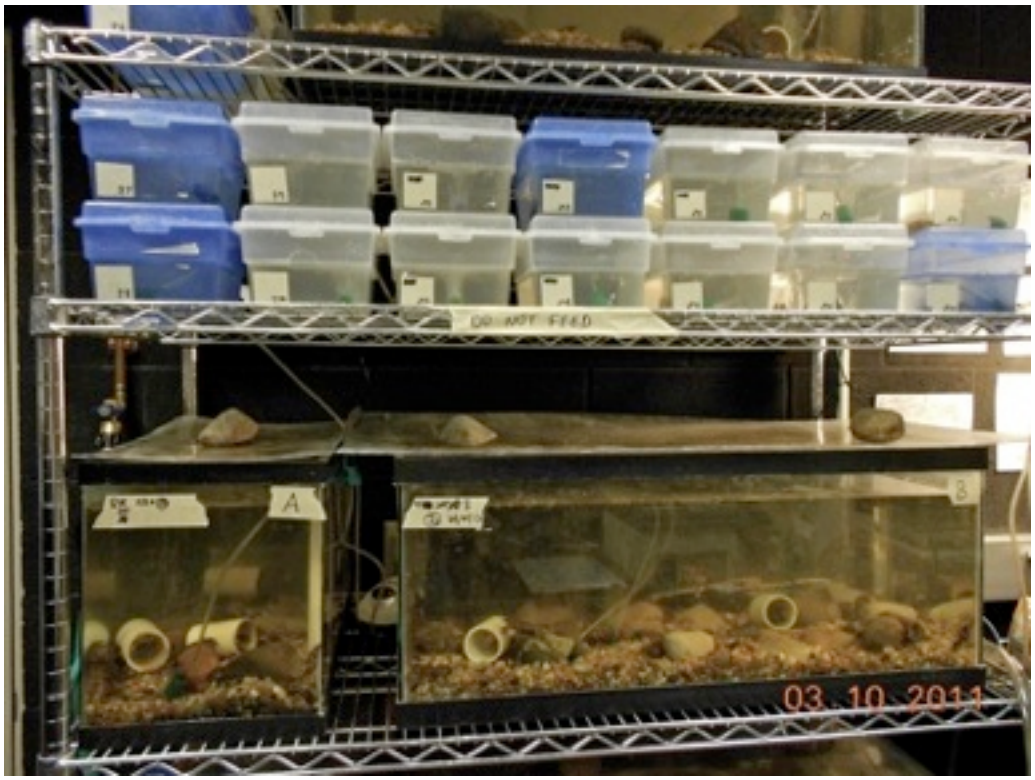


Figure 4.

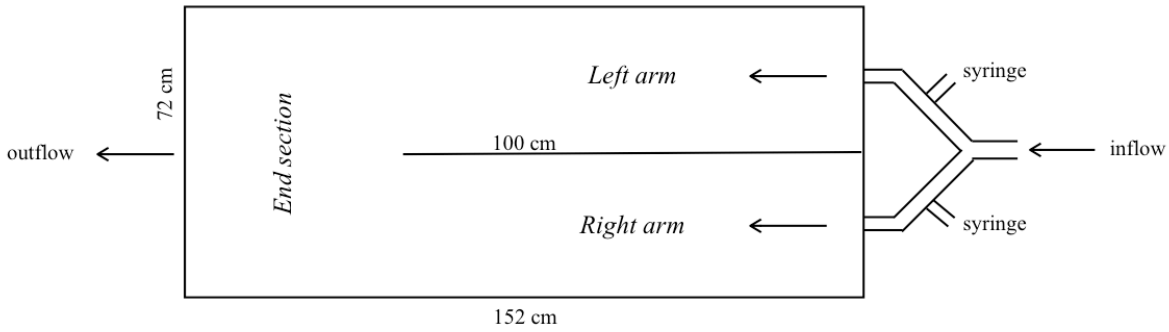

72 cm
outflow
End section
Left arm
100 cm
Right arm
syringe
syringe
inflow
152 cm

Figure 5.

Figure 6.

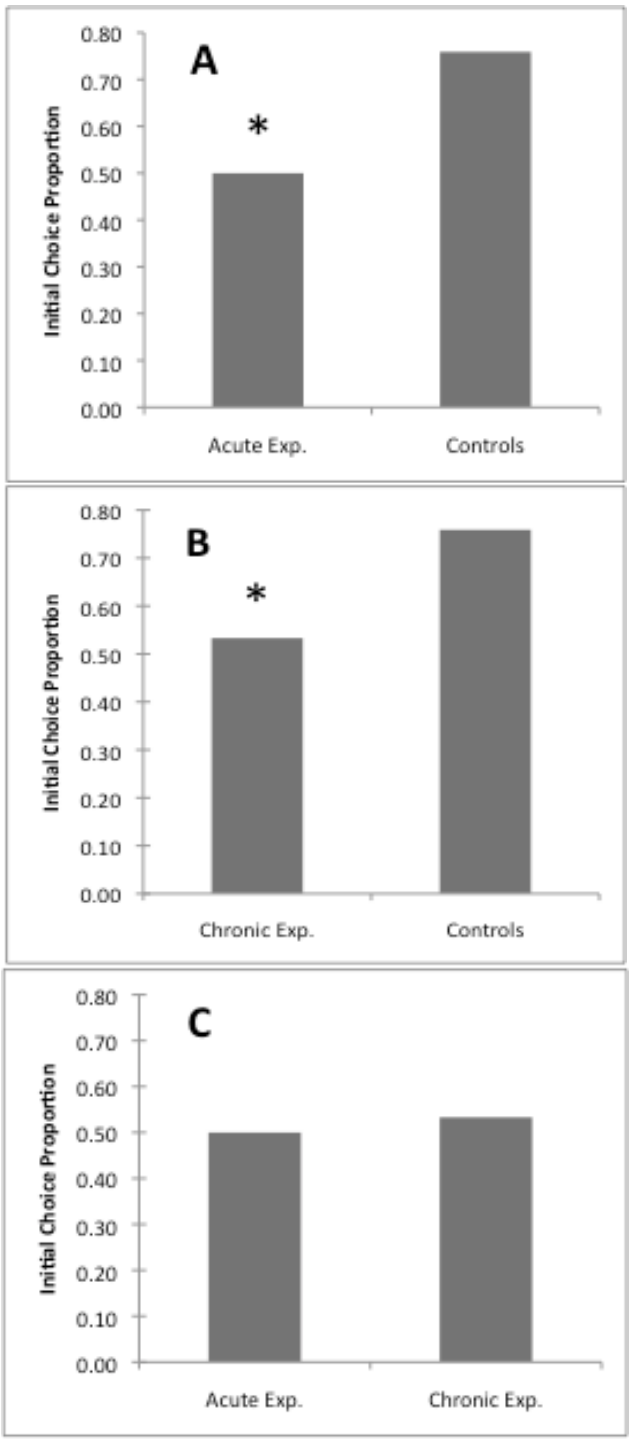
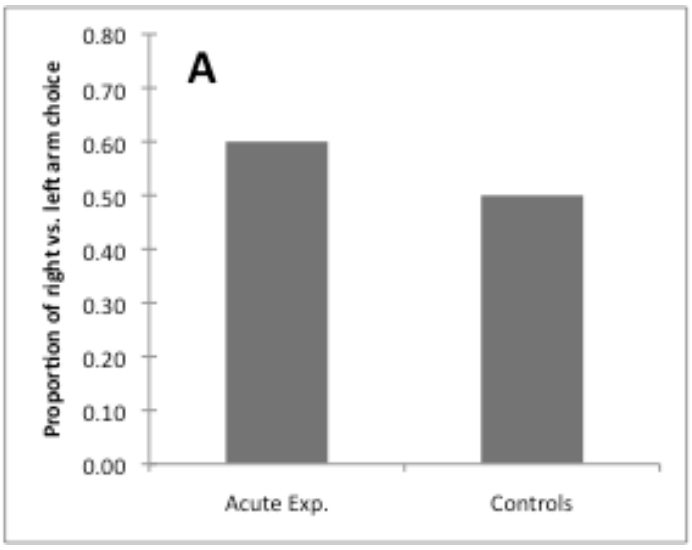

A
*
Initial Choice Proportion
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Acute Exp.
Controls
B
*
Initial Choice Proportion
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Chronic Exp.
Controls
C
Initial Choice Proportion
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Acute Exp.
Chronic Exp.

Figure 7.


A
Proportion of right vs. left arm choice
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Acute Exp.
Controls

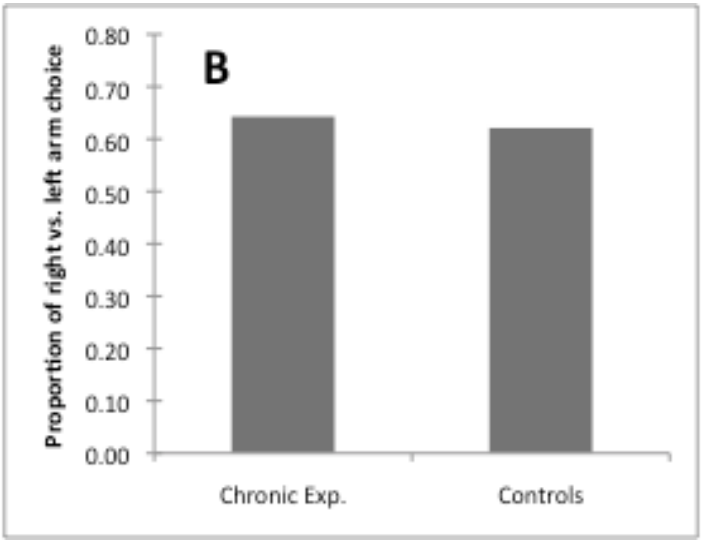

B
Proportion of right vs. left arm choice
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Chronic Exp.
Controls

Figure 8.

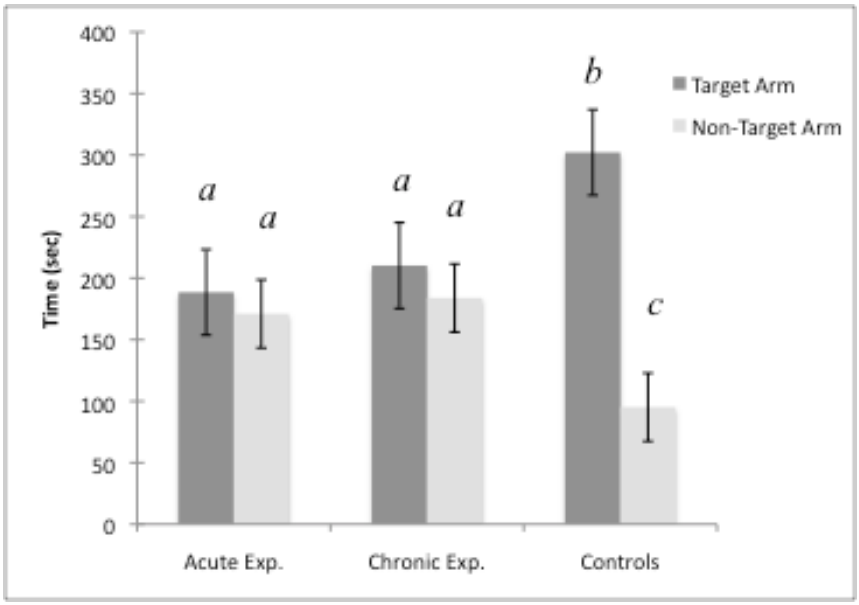

400
350
300
250
200
150
100
50
0
Time (sec)
a
a
a
a
b
c
Target Arm
Non-Target Arm
Acute Exp.
Chronic Exp.
Controls

Figure 9.