Chronic Effects of Nonylphenol on Reproductive Behavior, Physiology, and Development of Crayfish

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Abstract

Nonylphenol is a commonly used surfactant in a variety of industries. Nonylphenol shows an affinity for estrogen receptors, hence its classification as an endocrine disruptor and potential danger to reproductive success. Nonylphenol accumulates in aquatic environments and several studies have demonstrated reduced olfaction and impaired gonad development in a variety of species after exposure. Although acute studies have been performed, chronic exposure studies are limited. A total of 240 crayfish, *Orconectes propinquus*, consisting of 60 adult males, 60 adult females, 60 juvenile males, and 60 juvenile females were collected to locate a mate, electrophysiological recordings of olfactory neurons, and examined gonad morphology four months post-exposure. A behavioral assay was performed using a Y-Maze, electrophysiological recordings of antennules were obtained through use of the Backyard Brains Spikerbox, and individual weekly mass, molting events, mortality, and final gonad mass were all recorded during exposure. Statistical analysis on behavior data, electrophysiological recordings, and mass data were performed using a repeated measures ANOVA test. Gonad mass was analyzed using a one way ANOVA. Mortality data was analyzed using a Kaplan-Meier survival analysis. Exposing crayfish to varying sublethal concentrations of nonylphenol affects behavior, neuron responsiveness, and development. Observing both the behavioral and developmental effects of low, but chronic nonylphenol exposure, provides insight to its potential effects on crayfish populations and ecosystems.

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Chapter 1: Introduction

Introduction

Pollutants are toxic chemicals that adversely affect humans, animals, and the environment. Often these pollutants are persistent and can accumulate in environments, potentially causing residual effects on inhabitants. Nonylphenol (NP) is an organic compound that is the breakdown byproduct of alkylphenol ethoxylates, a class of persistent bioaccumulating chemicals. It remains a common non-ionic surfactant used for industrial, agricultural and domestic purposes like paints, pesticides, detergents, and plastics. It is not readily removed during wastewater treatment, as the ethoxylate group dissociates leaving the remaining alkylphenol backbone intact. NP accumulates in soil and in oxygen poor regions of lakes, rivers and streams, subsequently affecting the inhabitants of these areas. It is biologically active and acts as an estrogen mimic, binding to estrogen receptors. The detrimental physiological effects of nonylphenol have been observed in several species including rainbow trout, salmon, lobsters, crayfish and even humans. Specifically, it has been demonstrated to inhibit olfaction, diminish reproductive capabilities, and hinder development in a variety of organisms. Although many freshwater regions are below the recommended EPA legal levels, toxic and harmful effects have been observed at these concentrations. Typically, research is conducted on these chemicals at higher levels, therefore examination at sub-lethal lower concentrations is conducted less often. Crayfish are a critical part of an extensive trophic web, therefore examining the long-term effects of nonylphenol at

lower sub-lethal concentrations on behavior, physiology, and development contributing to their reproductive capacity is imperative to understanding the extent of NP's influence on ecosystems. Accumulation of nonylphenol in crayfish and transfer throughout the trophic levels can ultimately lead to potential infiltration into humans and adverse effects on human health.

Purpose

Observing the behavioral, physiologic, and developmental effects of variable nonylphenol dose exposure over an extended period of time will provide insight to the potential effect on crayfish populations and subsequent ramifications on ecosystems at current EPA level guidelines.

Scope

A population of 240 crayfish of the species *Orconectes propinquus*, consisting of 60 adult males, 60 adult females, 60 juvenile males, and 60 juvenile females were exposed to environmentally relevant nonylphenol concentrations over a four month time period to observe chronic effects on reproductive behavior, electrophysiology, and development. The scope of this study is limited by the population and species selected. The exposure period was limited to four months in an effort to observe long term effects that current research is lacking, while remaining within the time restraints of the project.

Assumptions

We assumed that crayfish would not demonstrate a side bias in behavior trials and that crayfish selected for each exposure group did not experience different exposures prior to initiation of the study. Prior research has shown that nonylphenol adversely affects the crayfish's ability to find food. Therefore we assumed that similar effects could occur when testing mate odor detection.

Hypothesis

I hypothesized that behavior, physiology and development will show dose dependent decreases in response to increased nonylphenol concentrations.

Research Questions

1) How will chronic exposure of crayfish to nonylphenol affect reproductive capacity as measured by behavior, physiology, and development?
2) Are changes to reproductive capacity concentration dependent?
3) Does age at onset of exposure affect reproductive capacity?
4) Are males and females differentially affected by nonylphenol exposure?

Chapter 2: Review of Literature

Sources of Nonylphenol

Nonylphenol is an alkylphenol that is commonly used non-ionic surfactant in a variety of industries. Its chemical structure is composed of hydrophobic and hydrophilic regions. This structure allows for polar and nonpolar substances to be easily miscible, making it an incredibly versatile chemical. As a result of this versatility, around 500,000 tons of alkylphenols are produced annually (Naylor et al., 1992). Industrially, it is used in paper mills and the production of plastics, paints, and lubricants. Alkylphenol ethoxylates, specifically Nonylphenol ethoxylates, are commonly used in herbicides and pesticides to increase their ability to adhere to leaf surfaces. However, runoff from irrigation systems or precipitation act as direct sources of nonylphenol contamination to surrounding watersheds. Domestically, NPs are found in a variety of fabrics, household detergents and cleaners. Food packaging materials and skin care products often contain significant levels of nonylphenol, acting to directly expose humans to any potential effects of the chemical (Soares, 2008). Although the majority of nonylphenol ethoxylates from households and industries are processed by wastewater treatment plants, nonylphenol is not removed. Rather it simply loses its ethoxylate group, preventing it from further acting as a detergent (Ying et al., 2002). In 2008, Soares found 60-65% of nonylphenol compounds that enter wastewater treatment plants will ultimately enter the environment at concentrations of 0.790 µg/L in outflow effluent.

Nonylphenol Entering Watersheds

Wastewater treatment plants receiving waste water from urban areas have the highest documented nonylphenol levels, leading to increased concentrations that exit these urban wastewater treatment plants into surrounding watersheds (Soares, 2008). Upon exiting water treatment plants, nonylphenol settles out of the water column and accumulates in the water and sediments of the surrounding areas. Sediment NP concentrations are highest at downstream sites closest to wastewater treatment plant outflow. Sediment samples of NP in these areas have been found to be as high as 3000 μg/L. Higher temperatures also result in higher sedimentary nonylphenol concentrations due to increases in microbial activity breaking down nonylphenol ethoxylates, leading to increased nonylphenol accumulation in the adjacent water (Chen et al., 2014; Naylor, 1992). While NP enters the environment through wastewater, it can also enter watersheds from farm run-off and industrial dumping, bypassing water treatment plants altogether. Concentrations of NP in both soil and water samples increase during the wet season due to warmer temperatures, increasing microbial activity and higher levels of rainfall that resulting in more surface run-off, bringing with it high concentrations of NP found in herbicides and pesticides in agriculturally dominant regions (Xu et al., 2015). NP contaminated run-off can then enter surrounding rivers and lakes which can average 0.10.8mg/L of nonylphenol (Soares, 2008). Nonylphenol can then be absorbed into the ground and this polluted groundwater can spread kilometers from the source, then taking decades to degrade. Moreover, septic systems that can leak into the environment have measured NP levels as high as at 1.2g/L (Soares, 2008). Nonylphenol is found to be most abundant in river water and still present when drinking water was tested at concentrations of 15-85ng/L (Chen et al.,

2014; (Soares, 2008). Nonylphenol has also been found in the atmosphere, as it is aerosolized by wastewater treatment plants, and then returns to aquatic ecosystems in the form of precipitation. It has been found in the air (110ng/m^3) and dust (2.58 µg/g) of households (Soares, 2008). With multiple possible avenues for nonylphenol to enter the environment, regulations and consistent monitoring of NP concentrations is necessary to maintain healthy ecosystems.

Environmental Regulations

Prior to strengthening of environmental benchmark criteria, nonylphenol concentrations in the Great Lakes groundwater ranged from 0.01 µg/L-0.92 µg/L in 1995 (Careghini, 2015). As of 2010, the Environmental Protection Agency (EPA) in the United States has two categories for exposure to nonylphenol. Acute exposure is defined as concentrations not exceeding 6.6 µg/L more than once every three years. Whereas, chronic exposure is 1.7 µg/L over four days not more than once every three years (U.S. EPA, 2010). After being implemented, mean NP levels in the Great Lakes from 2010-2013 were 0.842-1.16 µg/L in urban areas and 0.685-0.86 µg/L in nonurban areas. However, concentrations as high as 5.1 µg/L in urban regions and 6.6 µg/L in nonurban regions were documented (Baldwin et al., 2016). While these regulations tackle aspects of NP pollution, they do not account for the extended period of time NP subsists within the environment as the half-life of nonylphenol has been documented to range from 58 days to 60 years (Ying et al., 2002; Shang et al., 1999). Elsewhere, the European Union has instituted a voluntary ban on nonylphenol with the emergence of overwhelming research. Environment Canada requires water levels to be below

0.7 µg/L for indefinite chronic exposures, yet, the U.S. does not have an equivalent standard. The long-term persistence of nonylphenol in aquatic environments necessitates its consistent monitoring as aquatic organisms are persistently exposed to NP within their ecosystem and has the potential to bioaccumulate. By testing aquatic organisms at levels at or below Canada's current regulations, we can also help establish if these guidelines are reasonable to adopt in the United States or perhaps whether these regulations should be re-evaluated.

Bioaccumulation

Nonylphenol has been found to be most abundant in river water and sediment and is found at levels higher than other potentially ecologically hazardous chemicals (Chen et al., 2014). Nonylphenol's presence has significant implications for exposed aquatic species, as it has been shown to bioaccumulate. This results when an organism absorbs a substance and lacks the capacity to completely excrete it, resulting in subsequent accumulation within various tissues. Bioaccumulation can occur from dietary intake, respiration and dermal absorption (Mackay and Fraser, 2000). Nonylphenol is easily acquired by many fish species from the water, sediment, and particulates. For example, a significant correlation exists between high environmental concentrations and accumulation in tilapia tissues (Lee et al., 2015; Chen et al., 2014). Various concentrations of NP were also found in different species of fish that could be correlated to feeding modes, and/or absorption or removal capacity. Demersal (bottom dwelling) fish showed higher NP levels than pelagic (mid-water) fish (Xu et al., 2015). Moreover, accumulation of NP was higher in wild fish than farmed fish in both freshwater and marine species. The species with the highest accumulation of NP was wild freshwater fish (Lee et al.,

2015). While NP bioaccumulates, it may do so differentially in various organ systems. Bioaccumulation of nonylphenol was 5-20 times higher in eggs and gonads than in muscle tissue. Once deposited in tilapia eggs, nonylphenol was found to be transferred to offspring, potentially threatening reproductive success (Chen et al., 2014). In addition, any organism that absorbs NP can pass it onto higher trophic levels via consumers, resulting in consumers near the top of trophic webs accumulating more concentrated chemical doses. Organisms that bioaccumulate can be used to monitor water and sediment quality related to environmental contamination (Mackay and Fraser, 2000). It does not only accumulate in animals, but also in plants. Nonylphenol accumulation levels were recorded in carrots, pumpkins, apples and citruses, which could lead to human consumption and bioaccumulation of NP (Careghini, 2015).

Endocrine Disruption

Due to its similar structure to estrogen, nonylphenol shows an affinity for estrogen receptors, hence its categorization as an endocrine disruptor (Figure I). Endocrine disruptors diminish development and overall fitness of exposed organisms (Xu et al., 2015). Nonylphenol found in river water samples was first calculated to have low estrogenic activity, however, estrogenic activity is higher than previously estimated as tests typically underestimated estrogenic activity (Leusch et al., 2010). Some estrogenic molecules have increased bioactivity or estrogenicity, however, all follow similar trends. Differences in intensity/reactivity would suggest an array of mechanisms that are molecule specific ranging from genomic, mitotic related non-genomic, or receptor-isomer interactions (Leusch et al., 2010) Balakrishnan et al. found NP causes increases in genetic mutagenicity in a time dependent manner (2014). Watson

identified multiple non genomic mechanisms all affected by low dose nonylphenol ethoxylate exposure including PRL release, cell proliferation, calcium influx, and the activation of MAP kinases (2009). Routledge and Sumpter found that both the position and branching of the alkyl group of the nonylphenol ethoxylate affected estrogenicity. Para position and tertiary branched isomers showed the greatest estrogenic activity (1996).

Nonylphenol acts by competitively binding to receptors for 17β-estradiol, a hormone that regulates the growth and development of female sex characteristics and accessory sex organs. The effects of NP as an endocrine disruptor are not limited to binding estradiol receptors as tissue specific effects have been documented (Soares, 2008). CYP1A1 and HSP70 expression were significantly higher in wastewater groups. HSP70 is a protective protein involved in repair for cells exposed to environmental stressors, indicating organism stress when exposed to wastewater (Chen, 2016). Estradiol regulates and increases expression of CYP1A1, which is involved in the metabolism of foreign agents, the byproducts of which contribute to carcinogenesis (Go et al., 2015). Increased CYP1A1 expression indicates potential harmful implications for exposed organisms. NP also increases the expression of aromatase, estrogen receptor, and vitellogenin (VTG) genes (Xu et al., 2015). Aromatase converts androgens to estrogens and VTG is an egg yolk protein. Increased expression of either is commonly referred to as feminization of the organism. Hepatic VTG expression in both male and female fish is significantly increased following NP exposure, further suggesting feminizing endocrine disruption (Chen, 2016). Aromatase genes and isomers are up-regulated in juvenile zebra fish exposed to NP, while VTG mRNA up-regulation occurred upon activation of estrogen receptor beta. Age-related changes are necessary to consider when observing potential modifications

that could impact population dynamics, as these changes occurred in the first fry stage of fish (Xu et al., 2015). Fish exposed to water containing higher NP concentrations had a significant higher number of eggs relative to controls (Chen, 2016). Normally, gonad inhibiting hormone (GIH) inhibits estrogen levels and downregulates vitellogenin, but nonylphenol significantly decreases GIH levels which could directly impact the number of eggs (Li et al., 2015). Nonylphenol also interferes with male development, exhibiting anti-androgenic activity related to the upregulation of aromatase and decreases in GIH.

Humans

While animal exposure to nonylphenol has been documented, the effects on humans increase attention on the issue. Nonylphenol is shown to bioaccumulate, therefore exposure of lower trophic organisms such as fish and crayfish will lead to increased levels in higher organisms, such as humans. Average daily adult human intake of nonylphenol from food sources is 0.067-0.370 µg/kg, while the average intake from drinking bottled water daily is 0.36-0.60 µg (Careghini, 2015). When exposed to these NP concentrations, humans can only excrete 10% of ingested NP, the other 90% is absorbed in human tissues (Soares, 2008). Prenatal exposure has been observed as well. Placental exposure to NP affected cytokine production in the first trimester. GM-CSF, IFN gamma, IL-1 beta, IL-4 and IL-10 levels were increased in nonylphenol exposed samples relative to controls and TNF alpha was suppressed at low level exposure and stimulatory at high concentrations (Bechi et al., 2010). Changes in cytokine production could lead to varying expression of hCG, affecting placentation, fetal growth disruption, and other disorders (Bechi et al., 2010). The effects of human exposure to NP has

been associated with younger puberty onset in females and increased breast tumor proliferation (Chen et al., 2009; Soto et al., 1991). Chronic NP levels originating from human activity and stagnant EPA guidelines, could create a ripple effect through the trophic levels of various ecosystems resulting in potentially ominous effects on human reproductive capabilities.

Crayfish as a Model Organism for Nonylphenol Exposure

Various nonylphenol effects have been studied in both aquatic vertebrates and invertebrates, including crayfish. Whether emitted from farm run off or water treatment plants, nonylphenol accumulates in deoxygenated regions of fresh bodies of water, the ideal benthic habitat for a crayfish. This increases the NP exposure for crayfish both short and long term. NP concentrations can vary seasonally based on differences in annual climate and subsequent rainfall (Chen et al., 2014). Crayfish mating seasons are typically during the spring and fall, which overlaps with the rainy seasons, any increases in water concentrations of nonylphenol could ultimately affect their ability to reproduce. Negative effects on reproductive capacity would likely lead to decreases in crayfish population numbers, which could have drastic ramifications within ecosystems. Crayfish act as a food source for humans and a primary food source for over forty vertebrates (VanArman, 2011). They are also predators that consume a wide variety of plants and invertebrates. The polytrophic role as both predator and prey makes crayfish presence in ecosystems highly influential through various trophic levels and are thus considered a keystone species (Usio, 2000; Helms and Creed, 2005). Crayfish also act as a significant regulator of the carbon cycle, releasing energy for organisms in higher trophic levels (Usio, 2000). Effects on crayfish reproduction and subsequent changes in population numbers

would therefore impact all levels of the trophic web. With the tight correlation between crayfish and many other species, increased bioaccumulation of NP in crayfish would then extend throughout the trophic web as well. Studies show that benthos like crayfish are a likely source of entry of endocrine disruptors into the trophic web (Xu et al., 2015).

When compared to other organisms, crustaceans were the most sensitive to NP exposure, likely due to the high NP concentrations in their benthic habitat and their increased bioaccumulative nature (Xu et al., 2015). Paired with their importance in maintaining the overall health of ecosystems, investigating the potential implications of chronic exposure to the species is imperative to preventing catastrophic ecological effects. Crayfish reproduction is multifaceted and requires successful courtship behavior, functional physiological systems, and healthy development. Impairment of any of these three tiers would weaken the population. In vivo experiments on various model organisms measure NP impacts on survival, growth, secondary sexual development, organ weight, plasma vitellogenin, sexual steroids in the plasma, fecundity, gamete viability and histology. These parameters are crucial for accurately estimating environmental activity and eventual effects on population dynamics (Soares, 2008).

Nonylphenol Effects on Reproductive Behavior

Behavior of many aquatic species has been altered after exposure to various environmental contaminants. Pollutants can affect behavior by interfering with multiple mechanisms. Neurological development, sensory receptors, or endocrine systems are all potential mechanisms of impairment (Weis, 2014). Behavior of many aquatic species has been altered after exposure to various environmental contaminants. Various pesticides have been shown to interfere with and inhibit acetylcholinesterase in fish and lead to changes in mobility

(Weis, 2014). Similar changes in mobility after exposure to nonylphenol would suggest perhaps an underlying mechanism for movement impairment in aquatic organisms. While embryonic exposure to pesticides has demonstrated reduced spatial discrimination, it may indicate that alterations in development could differentially affect behavioral outcomes of exposed organisms (Weis, 2014). Nonylphenol has already been shown to significantly affect reproductive behavior in several fish species. Fathead minnow male larvae exposed to nonylphenol ethoxylates had reduced capacity in competing for spawning sites, while 0.5µg/L of 4-nonylphenol caused impaired social interactions of juvenile killifish, and 1-2µg/L caused avoidance behavior (Weis, 2014). Weis expresses the need for future research to connect behavioral effects to changes in ecology, especially reproductive behavior, and its importance in population success (Weis, 2014).

Species with impaired reproductive behavior leads to decreased reproduction and subsequent declines in a population. Therefore as a keystone species, the success or failure of crayfish populations directly influences ecosystem success. Crayfish use chemical signals in their urine for communication for courtship and/or when establishing social dominance. Urine is released through anterior facing nephropores. Once released, it is carried through water currents that can be manipulated by crayfish (Bergman et al. 2005). For detection of odorant or chemical signals, crayfish draw water towards their antennules, which are the primary olfactory organ of the crayfish, responsible for chemoreception within the environment.

Upon reaching sexual maturity, crayfish will actively seek a mate, a behavior which is under hormonal control and influenced by water temperature and seasonal light (Yazicioglu, 2016). Reproductive behavior in crayfish is initiated when a receptive female displays

aggression and releases urine. Female crayfish urine contains a female sex pheromone which increases reproductive behavior in males (Breithaupt, 2011). Females readily release urine in reproductive interactions, while male urine pheromone release is less consistent when courting. Males will respond to female odor, yet, female crayfish do not act differently to male/female conspecifics on odor alone. Once detecting female urine, a male will engage in aggression and attempt to mate with her. If the male can overcome female resistance, he copulates with her and deposits spermatophores under her abdomen for future fertilization of eggs (Breithaupt, 2011). While reproductive behavior has been well characterized under normal conditions, pollutants have been found to have significant effects on the reproductive behavior of decapod crustaceans. Studies have shown that male crustaceans exposed to pollutants were less effective at locating females (Olsen, 2010). Not only were exposed crustaceans less successful at finding a mate, they were searching for a mate less. In mating efforts, pollutant exposed male amphipods searched for females less than unexposed males (Krang, 2007). The effect of chronic nonylphenol exposure on crayfish reproductive behavior has yet to be studied. However, with many studies documenting disruptions in the reproductive behavior of aquatic organisms after exposure to pollutants, pesticides, and specifically nonylphenol ethoxylates, studying the effects specifically on crayfish is warranted. Following reproductive behavior of both male and female crayfish of various stages in development at nonylphenol concentrations below the EPA guidelines will reveal if 1) the ability of crayfish to find a mate changes due to chronic exposure or 2) if they are less likely to search for a mate following exposure.

Nonylphenol Effects on Reproductive Physiology

Studies suggest that water pollutants can interfere with olfactory detection and communication, impacting behavior necessary for reproductive success (Olsen, 2014). As crayfish reproduction requires finding a mate which relies on detection of chemical signals via chemoreception, any impairment in this system could result in decreased courtship. Exposure to chemicals can cause cell death of olfactory cells, changes in sensory cells, and inhibit growth and differentiation of neural stem cells which would lead to impaired chemoreception (Weis, 2014; Soares, 2008). Many species rely on olfaction in detecting sex pheromones for reproductive success (Olsen, 2014). A plethora of studies have shown impaired capacity for olfaction after pollutant exposure resulting in hindered ability to locate food and inability of males to locate females (Olsen, 2010). Impaired olfaction is theorized to be the cause of reduced homing ability, reproductive capacity, and response to female pheromones in male salmon and rainbow trout (Saucier et al., 1991; Hara, 1992; Moore and Waring, 1996; Scholz et al., 2000). The olfactory system is particularly vulnerable to dissolved substances as these external structures are constantly exposed. Olfactory receptor neurons can also bind and transport contaminants to the brain causing subsequent neural damage (Weis, 2014). In fact, exposure to pesticides has been shown to reduce olfactory electrophysiological responses in fish (Olsen, 2014). Specifically, responses of olfactory epithelium of atrazine exposed salmon were significantly reduced in response to serine and urine (Moore, 2007). Reductions in olfactory electrophysiological responses indicate decreased chemoreceptive sensitivity, which would lead to decreases in behavior requiring olfaction such as reproduction and finding food. Fish are less likely to have chronic olfactory impairment as they have an increased olfactory cell

turnover rate relative to crustaceans, which only replace olfactory cells when molting (Hallberg and Skog, 2010).

Crayfish rely on olfaction for a variety of functions including navigation to food sources and mates. Consequently, any effect on olfaction would significantly impact their capacity for survival and reproduction. Olfactory signals are carried via olfactory neurons within the crayfish antennules to the brain to be interpreted. Several studies have shown the reduction in olfaction from nonylphenol exposure and the diminished ability for crayfish to find food. Crayfish exposed to nonylphenol for 1 and 4 days demonstrated a reduced capacity to find food relative to controls. Exposure affected the total amount of time crayfish spent correctly identifying food smell. Exposed crayfish spent equal time in correct and incorrect regions of a Y-maze and significantly less time in the correct regions relative to controls (Page, 2013). Since crayfish also rely on olfaction in mating and courtship, impairment in this system would also lead to a decreased ability to locate a mate. The mechanism of how nonylphenol impairs the crayfish's ability to find food has yet to be fully determined. Measuring the field potentials of the antennules to various chemical stimuli could provide the mechanism that contributes to an inability to find food or a mate. There are several possible explanations for the effects of nonylphenol on behavior, but measuring field potentials could provide an explanation for the observed effects.

Nonylphenol Effects on Reproductive Development

Alkylphenols have been demonstrated to have a variety of effects, such as endocrine disruption, on vertebrates and crustaceans that result in sensory, growth, and reproductive

impairments (Cook and Moore, 2007). Fish embryos exposed to nonylphenol showed decreased survival and diminished development of secondary sex characteristics (Soares, 2008). The presence of alkylphenols has been shown to induce vitellogenesis, demonstrating the endocrine disruptive effects in the manner of feminization (Jones et al., 2000). Hayes et al. (2002) showed sub-lethal levels caused demasculinization and hermaphroditism in frogs. However, Chen et al. found no changes in fish gonad histology or gametogenesis relative to controls (2016). In mammals, the endocrine disruptive effects of similar molecules have been observed causing reproductive physiological complications (Stoker et al., 1999). In rats, increased nonylphenol exposure led to increased endometrial mitotic activity in females and decreased sperm motility and acrosome damage in males (Soto et al., 1991; Uguz et al., 2009). Nonylphenol also increases mammary gland cell proliferation and changes mitochondrial membrane permeability (Soares, 2008).

There are limited studies on endocrine disruptors in invertebrates, especially in early life stages, which could lead to not only defects in behavior, but dramatic reductions in reproductive ability later in life (Weis, 2014). A direct correlation of alkylphenol concentration and juvenile hormone activity has been documented in crustaceans. In lobsters, this increased activity may inhibit shell hardening after molting, consequently increasing vulnerability to disease or predators (Biggers and Laufer, 2004). Crayfish possess neuroendocrine X-organs, which inhibit molting, while epithelial Y-organs release ecdysone in preparation for molting (Longshaw, 2016). If nonylphenol disrupts either of these hormone related processes, changes in molting frequency would exist in exposed organisms. Molting typically occurs in response to normal growth when an organism outgrows its exoskeleton and must regenerate a larger shell.

Endocrine disruptors affecting molt frequency would potentially affect the growth of the organism. While environmental temperature typically influences growth rate, endocrine disruption could impact organism growth, an otherwise normal developmental processes (Longshaw, 2016). Low level NP concentrations has been found to enhance growth and reproduction in nematodes (Soares, 2008). While disruption of molting could lead to increased mortality and consequential effects on crayfish population dynamics, molting also impacts reproduction. When a female crayfish molts, all stored sperm are lost (Longshaw, 2016). Increases in molting frequency would therefore lead to decreased chances of fertilization and generation of zygotes. Examining molt patterns related to NP exposure could indicate one major pathway for disrupting development.

The presence or absence of hormones also influences development of an organism, and sex hormones specifically affect gonadal development. Behavioral and physiological changes in crabs have been documented resulting in impaired phototaxis and testis weight (Forward and Costlow, 1976; Lye et al., 2008). Due to the endocrine disruptive effects of alkylphenols observed in testis development and diminished sperm production in a variety of species, crayfish are likely to exhibit similar results when chronically exposed to sub-lethal nonylphenol concentrations. Using juvenile male and female crayfish exposed to nonylphenol allow for the examination of growth and development that may be contingent upon the amount and/or time exposed. At the end of exposure, measuring gonad size and observing any morphological changes may indicate an effect of impaired or exaggerated gonad development. If the data indicated either deviation, this would demonstrate an additional effect that could be dose dependent.

Chronic Exposure

While acute studies have been performed on a variety of species, to our knowledge chronic studies measuring the effects of varying concentrations of nonylphenol have yet to be performed. Nonylphenol does accumulate in a time dependent manner and extended exposure could reveal further implications to populations exposed at or below current EPA levels (Balakrishnan et al., 2014). The overall goal of my research was to examine the chronic effects of nonylphenol on crayfish courtship and factors that may influence reproduction. Due to the bioaccumulating nature of nonylphenol and the significant role crayfish play in the trophic web of various ecosystems, studying the effects on these organisms is essential. Due to its estrogen mimicking quality, nonylphenol could disrupt the drive or ability to find a mate, therefore it is important to compare the effects on males and females, as it is possible they could be affected differently. Testing the crayfish's ability to find a mate after nonylphenol exposure would help to categorically indicate that nonylphenol disrupts mating behavior and eventually population numbers. If the results indicate a difference in performance, chemoreception, molting rates, death rates, or overall growth of the organism, solutions could be specifically designed to target and protect the affected population.

Chapter 3: Methodology

Experimental Group Selection

A total of 240 crayfish, *Orconectes propinquus*, consisting of 60 adult males, 60 adult females, 60 juvenile males, and 60 juvenile females were collected by use of a seine net in the Little Rio Grande, a tributary of the Grand River in Michigan, between May and August of 2017. After collection, crayfish were sorted by sex and mass. Sixty juvenile male crayfish under 3 grams were then randomized into three groups of 20. This was repeated with sixty juvenile females. Sixty adult male crayfish over 6 grams and sixty adult female crayfish over 4.5 grams were each separately randomized into three groups of 20. Each group of 20 crayfish was assigned to one of three experimental groups and the initial mass of each crayfish was recorded. Crayfish were assigned an identification number that was written on the carapace with silver permanent marker to individual animals over the course of exposure and trials.

Exposure Set-up

Nonylphenol was purchased from Sigma-Aldrich. Due to its viscosity and hydrophobic qualities, 2.0 mL acetone was used as the vehicle to dissolve nonylphenol and later transfer to water in the experimental procedures. Prior to submersion in 2 mL acetone, the pipette exterior was cleaned with acetone as residual nonylphenol on the pipette would significantly alter doses. Nonylphenol-acetone solution was stored as stock solutions. Stock solutions were agitated prior to being added to tanks. As acetone was essential for nonylphenol solutions,

control tanks used vehicle acetone within water at analogous concentrations for the experimental trials. Survival and behavior was documented for all concentrations.

Each experimental group of 20 crayfish was randomly assigned one of the concentration categories as control, low level sub-lethal nonylphenol exposure, or high level sub-lethal nonylphenol exposure. Control crayfish were exposed to the vehicle acetone. The low level sublethal concentration was at 0.15 µg/L of nonylphenol with the added acetone. The high level sub-lethal concentration was at 0.30 µg/L of nonylphenol plus acetone. Concentrations were determined as sub-lethal based on Environment Canada and previous findings by Swift et al. (2017). To maintain concentrations for each group and maintain the blindness of the study, three Erlenmeyer flasks labeled A, B, and C were filled with a stock solution of each exposure concentration. Upon cleaning each tank, 0.6mL of stock solution from the appropriate flask was added to 20L tanks and 0.3mL was added to 10L tanks maintain consistent exposure. Crayfish that survived for the full course of the experiment were exposed for four months as the chronic exposure.

Experiment I: Behavioral Assay

In an effort to measure the ability of crayfish to find a mate, crayfish were individually placed in a Y-maze with water inflow from two arms towards a common base that was the location of water outflow and initial crayfish placement. Trials were run on 12 crayfish from each group (adult male, adult female, juvenile female, and juvenile male) and exposure (control, low, high) once a week for eight weeks for a total of 144 trials per week. Behavior trials began 7 days after exposure to respective nonylphenol concentrations or vehicle agent.

Tank for Behavior Assay

Similar to the design used by Page et al. (2013) and Adams et al. (2003), a modified Ymaze tank was used for these trials. The tank was rectangular in shape, was constructed from black acrylic, and measured 152 cm long, 72 cm wide, and 15 cm deep. It contained three regions: left arm, right arm, and neutral base. The arm divider was 100 cm long, which gave each arm a width of 36 cm. 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 Tconnecter 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, where a syringe pump was connected. The outflow was at the opposite end of the tank, in the neutral base. 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.

Stimuli for Behavior Assay

The stimulus used to measure the ability of crayfish to find a mate was water containing urine of the opposite sex, as courtship chemical signals are delivered via crayfish urine release. Water containing crayfish urine was collected from tanks containing 7-10 male or female conspecifics. These crayfish were fed prior to introduction to the urine collection tanks, but not

during their time in the tanks. This removed any potential confounding effects presented by a food odor in the water used as the attractive opposite sex stimulus. Water containing urine was collected after a minimum of 12 hours after the 7-10 crayfish were introduced to the collection tank. For all male trials, water containing female urine was used and vice versa for female trials.

Behavior Trials

Water speed was measured in both the right and left arms of the Y-maze prior to the initiation of trials. Water speed was maintained between 9.5 cm/s- 10.5 cm/s. For each trial, crayfish had a minimum of a 10-minute pre-acclimation period, where crayfish were isolated in preparation for the behavior assay. This was followed by a 10-minute acclimation period where the crayfish was placed in the neutral base of the maze while in a clear slotted plastic cage, allowing it to acclimate to the tank while not obstructing visual or olfactory stimuli. This acclimation period was followed by presentation of a single 3 mL primer burst release of urine water of the opposite sex by injecting the scent into either the right or left arm of the Y-maze one minute before initiation of the trial. The arm of urine odor injection was alternated between left and right arms for each trial. Upon initiation of the trial, urine containing water was delivered with the use of a syringe pump at a rate of 3 mL/min and crayfish were released from the plastic cage and allowed to explore the y-maze tank. Trials were recorded with a video camera for ten minutes. Videos were cataloged and analyzed by recording the number of visits to each arm of the maze (correct/incorrect arm), total time spent in correct arm, incorrect arm, or the base, and total time in an arm (sum of correct and incorrect) relative to time spent

in the neutral base. Video analysis was done by other lab members that were unaware of the experimental design to eliminate the potential for bias, creating a blinded study. The behavioral assay was repeated weekly with each group over the course of the eight-week exposure duration.

Experiment II: Electrophysiology Assay

In order to determine a potential mechanism of observed behavior, electrophysiological recordings of olfactory field potentials generated by neuron firing from the antennules were measured and recorded for both adult male and female crayfish that survived four months of exposure. Neurophysiological recordings from the antennules were made using the Backyard Brains Spikerbox. The Spikerbox allows for basic extracellular recordings from the neurons in the antennules. The three-stage amplifier is band-pass filtered between 300–1300 Hz and contains a speaker to make the neural activity audible. An output port was used for the computer recording with ~900× amplification. Neural data for these experiments were recorded using an open-source Backyard Brains app. The electrodes interfacing with the invertebrate preparations consist of two small #15 beading needles (0.25 mm diameter) soldered to a standard 24-gauge stranded speaker wire. The electrodes have a typical DC resistance of 0.3 Ω and a 1 kHz impedance of 20–30 kΩ (Marzullo and Gage, 2012).

Crayfish were chilled for 5 minutes prior to recordings to reduce motor activity. The antennules were removed and a neuron recording electrode was placed in each end of the antennule. Electrical signals were recorded in response to three stimuli. A resting field potential recording was performed without any stimulation. A second recoding was performed when

water was applied to the distal end of the antennule with a clean paintbrush for approximately 5 seconds and then removed. Once signal returned to initial resting state, a food slurry (0.29 grams of food in approximately 3 mL of water) was applied with a second paintbrush to the distal end of the antennule for approximately 5 seconds. This procedure was repeated with adult male and adult female crayfish in the control, low level nonylphenol exposed, and high level nonylphenol exposed groups. Juveniles were not used as the width of the antennules were too small for the recording electrodes.

Experiment III: Development Data Collection

After being sorted into experimental groups, initial masses were recorded. Crayfish masses were recorded weekly. If an individual crayfish molted, the date of the molting event was recorded. Death dates were also recorded to track mortality. Crayfish that expired during exposure had their final mass recorded and gonads extracted. Using dissecting scissors the carapace was removed, and the ovary (plus eggs) or testis were carefully removed with a forceps and isolated. The mass of each gonad was recorded. Gonad mass was compared to total crayfish mass to provide a gonad to total mass ratio for further analysis. After the fourmonth exposure, remaining crayfish were euthanized, massed, and gonads removed.

Analysis

Statistical analysis on behavior data, electrophysiological recordings, and mass data were performed using a repeated measures ANOVA test. Gonad mass was analyzed using a one way ANOVA. We checked that the assumptions were met in each case. Normality was tested

using Sharpio-Wilk and Kolmogorov-Smirinov tests. There were some concerns about normality in a few cases. For electrophysiology, the data underwent a square root transformation to meet the assumptions and improve normality of the residuals. We picked a covariance structure, (Auto-regressive, Compound Symmetry, or Diagonal), with the lowest values for AIC, AICC, and BIC using the democratic method. In instances where type III tests of fixed effects reported significant differences, Post Hoc tests were performed using the Bonferroni test for multiple comparisons.

Mortality data was analyzed using a Kaplan-Meier survival analysis. Deaths occurring within 24 hours of a molting event were classified as censored. This controlled for the possibility that nonylphenol exposure affected molting, allowing survival to be independently analyzed.

Chapter 4: Results

Experiment I: Behavior

1.1 *Percent of Time Correct*

Behavior trials were analyzed to determine the percent of time spent in the correct arm (arm with scent) relative to total time in an arm (correct or incorrect arm). Results are reported as a percent of time choosing an arm spent in the correct arm.

1.1.1 Adult Males

Low-level exposed adult males spent 30.0% on the correct side, high-level spent 30.4%, and controls spent 31.1%. Exposure group, time exposed to nonylphenol, nor the interaction of these two variables show a significant impact ($p>0.05$) on the percent of time adult males spent in the correct arm (Table I).

1.1.2 Adult Females

The interaction between exposure group and week has a significant impact on the mean percent of time adult females spent in the correct arm ($p=0.031$, Table II). Though the interaction was significant, a post-hoc analysis did not reveal a significant difference between any of the group by week combinations.

1.1.3 Juvenile Males

Exposure group had a significant effect on the mean percent of time juvenile males spent in the correct arm ($p=0.007$). The other variables had no significant impact (Table III). Pairwise comparison of exposure groups reveals the high and low exposure groups are both significantly different from the control group ($p=0.010$ and $p=0.023$ respectively). High exposure and low exposure groups are not significantly different from each other ($p=1.00$, Table IV). The low and high exposure groups are significantly lower (95% CI [22.789%, 30.514%]; [22.640%, 29.818%] respectively) than the control group (95% CI [30.289%, 37.265%], Table V). These results are depicted in Figure II.

1.1.4 Juvenile Females

Low-level exposed juvenile females spent 28.4% on the correct side, high-level spent 25.3%, and controls spent 28.2%. Exposure group, time exposed to nonylphenol, nor the interaction of these two variables show a significant impact ($p>0.05$) on the percent of time juvenile females spent in the correct arm (Table I). (Table VI).

1.1.5 Comparison by Sex

Low-level exposed adult females spent 30.2% while males spent 30.0%. High-level exposed adult females spent 26.5 % while males spent 30.4%. Adult female controls spent 29.6% while males spent

31.1%. Low-level exposed juvenile females spent 28.4% while males spent 26.6%. High-level exposed juvenile females spent 25.3% while juvenile males spent 26.0%. Juvenile female controls spent 28.2% while juvenile males spent 33.7%. Sex does not show a significant impact ($p>0.05$) on the percent of time spent in the correct arm, as male and females spent similar amounts of time in the correct arm (Table VII).

1.1.6 Comparison by Age

While age does not have a significant impact on the percent of time spent in the correct arm ($p>0.05$), exposure group does have a significant impact ($p=0.018$, Table VIII). A post-hoc analysis shows a significant difference in pairwise comparisons of the high level exposure group relative to controls in both the adults and juveniles($p=0.014$, Table IX). The high level exposure group spent significantly less time in the correct arm relative to controls (Table X). These results are depicted in Figure III.

1.2 Percent of Time Choosing an Arm

Behavior trials were analyzed to determine the percent of time spent choosing an arm (sum of time in correct and incorrect arm) relative to time in the neutral base. Results are reported as a percent of total time spent choosing an arm.

1.2.1 Adult Males

Low-level exposed adult males spent 60.6% of the trial choosing a side, high-level spent 59.8%, and controls spent 61.6%. Exposure group, time exposed to nonylphenol, nor the interaction of these two variables show a significant impact ($p>0.05$) on the percent of time adult males spent in an arm (Table XI).

1.2.2 Adult Females

The interaction between exposure group and week has a significant impact on the percent of time adult females spent choosing an arm (Table XII). Pairwise comparison of exposure groups by week reveals a significant difference in week two between low level exposure relative to controls and high level exposure groups ($p=0.001$, $p=0.050$ respectively, Table XIII). In week two, the low level exposure group spends significantly less time choosing an arm (95% CI [38.004, 53.659]) than controls (95% CI [60.113, 75.769]) and the high level exposure group (95% CI [51.741, 67.397], Table XIV). Within the low level exposure group, week two (95% CI [38.004, 53.659]) is significantly lower than week one and week four ($p=0.003$, 95% CI [59.944, 73.075]; $p=0.015$, 95% CI [57.518, 69.118] respectively, Table XIV and Table XV). These results are depicted in Figure IV.

1.2.3 Juvenile Males

The interaction between exposure group and week has a significant impact on the percent of time juvenile males spent choosing an arm (Table XVI). Pairwise comparison of exposure groups by week reveals a significant difference in week six between control and low level exposure groups ($p=0.008$, Table XVII). In week six, the control group spends significantly more time choosing an arm than low level exposed groups (95% CI [61.288, 77.578]; [44.133, 60.423], Table XVIII). These results are depicted in Figure V. The control and low level exposure groups do not show significant changes in time spent choosing an arm over time ($p>0.05$). The high level exposure group showed significant differences over time ($p=0.004$, Table XVIII and Table XIX). For every week of exposure, the control and low level groups spend at least 50% of the trial exploring arms rather than in the neutral base (95% CI $\geq$ 50%), while the high level exposure group spent the majority of the trial in the neutral base (95% CI [18.070, 45.205]; Table XVIII). These results are depicted in Figure V.

1.2.4 Juvenile Females

Week has a significant impact on the mean percent time juvenile females spent choosing a side ($p= 0.028$). The other variables had no significant impact (Table XX). Post-hoc analysis revealed a

change in percent of time choosing an arm by week tested. Week one was significantly different from weeks two, three, six, and eight ($p<0.05$, Table XXI). In week one, juvenile females spent significantly less time in an arm and more time in the neutral base than in the subsequent weeks (95% CI [39.286, 55.246], Table XXI and Table XXIII). These results are depicted in Figure VI.

1.2.5 Comparison by Sex

Low-level exposed adult females spent 57.1% while males spent 60.6%. High-level exposed adult females spent 57.4% while males spent 59.8%. Adult female controls spent 58.7% while adult males spent 61.6%. Low-level exposed juvenile females spent 55.7% while males spent 56.8%. High-level exposed juvenile females spent 56.0% while juvenile males spent 53.6%. Juvenile female controls spent 54.7% while juvenile males spent 58.8%. Sex does not show a significant impact ($p>0.05$) on the percent of time spent in an arm, as male and females spent similar amounts of time in choosing an arm relative to the amount of time in the base (Table XXIII and Table XXIV).

1.2.6 Comparison by Age

Age has a significant impact on the mean percent time spent choosing a side ($p= 0.012$, Table XXV and Table XXIII). The impact of age is significant for all exposure groups ($p=0.012$, Table XXVI).

Low-level NP exposed adults spent 58.1% of time choosing a side, while juveniles spent 55.0%. High-level NP exposed adults spent 58.2% of time choosing a side, while juveniles spent 55.1%. Adult controls spent 60.5% of time choosing a side, while juveniles spent 57.4% (Table XXVII). Adults spent significantly more time in an arm than juveniles (Table XXVI). These results are depicted in Figure VII.

Experiment II: Physiology

Amplitude of spikes were measured at 0.2 second intervals for the initial 5 seconds of each stimuli (resting, water, or food) for each recording. The original data underwent a square root transformation to increase normality of residuals. The untransformed data is in Table XXVIII and all further analysis refers to the square root transformed data.

2.1 Adult Males

The raw electrophysiological recordings of antennules are displayed in Figure VIII, separated by exposure group. Stimuli are ordered resting, water, and food. The interaction between exposure group and stimuli has a significant impact on generated field potentials ($p<0.0001$, Table XXIX). The transformed mV values for each stimuli response by exposure group are listed in Table XXX. Pairwise comparisons reveals significant differences in generated field potentials to varying stimuli dependent on exposure group (Table XXXI). In male controls, generated field potentials

are significantly higher when exposed to food than water ($p<0.0001$) and water is significantly higher than resting ($p=0.0002$). In low level exposed males, water and food do not have significantly different generated field potentials ($p=0.7465$). The low level resting generated field potentials are significantly lower than water and food mV ($p=0.0007$ and $p=0.0005$, respectively). High level exposed males had significantly higher generated field potentials to water than resting stimuli ($p<0.0001$) and resting generated field potentials were significantly higher than food ($p=0.0060$). Between exposure groups, there were no significant differences in the resting stimuli ($p>0.05$). No significant differences in generated field potentials exist between all three groups water stimuli and the low level exposure group food stimuli ($p>0.05$). The high level exposure group generated field potentials for food stimuli were significantly lower than most stimuli in all three groups with the exception of no significant difference between control resting values (Table XXXI). These results are represented in Figure IX.

2.2 Adult Females

The raw electrophysiological recordings of antennules are displayed in Figure X, separated by exposure group. Stimuli are ordered resting, water, and food. The interaction between exposure group and stimuli has a significant impact on generated field potentials ($p=0.0006$, Table XXXII). The transformed mV values for each stimuli response by exposure group

are listed in Table XXXIII. Pairwise comparisons reveals significant differences in generated field potentials to varying stimuli dependent on exposure group (Table XXXIV). In female controls, generated field potentials are significantly higher when exposed to food than water ($p=0.0004$) and water is significantly higher than resting ($p=0.0118$). In low level exposed females, water and food do not have significantly different generated field potentials ($p=0.3494$). The low level resting generated field potentials are significantly lower than water and food mV ($p=0.0005$ and $p=0.0004$, respectively). High level exposed females had significantly higher generated field potentials to water than resting stimuli ($p=0.0006$) and resting generated field potentials were significantly higher than food ($p=0.0095$). Between exposure groups, there were no significant differences in the resting stimuli ($p>0.05$). No significant differences in generated field potentials exist between all three groups water stimuli and the low level exposure group food stimuli ($p>0.05$). The high level exposure group generated field potentials for food stimuli were significantly lower than most stimuli in all three groups with the exception of no significant difference between control resting and water values (Table XXXIV). These results are represented in Figure XI.

2.3 Sex Comparison

Male and female controls do not show significant different generated field potentials by stimuli (Figure XII). Low level exposed male and female generated field potentials for resting are not significantly different. While low level exposed female water and food generated field potentials are significantly higher than low level exposed male water and food values, they follow the same trend. Low level exposed groups' generated field potentials for food and water are not significantly different (Figure XIII). High level exposed males and females do not show significant different generated field potentials by stimuli (Figure XIV).

Experiment III: Development

3.1 Survival

Survival of animals was tracked in number of days exposed. Death events that occurred within 24 hours of a molting event were classified as molt related deaths and were censored. (Molting frequency by exposure group is analyzed in section 3.4).

3.1.1 Adult Males

Nonylphenol exposure did not show a significant impact ($p>0.05$) on the survival of adult males (Table XXXV). Cumulative survival by exposure group is displayed in Figure XV.

3.1.2 Adult Females

Nonylphenol exposure did not show a significant impact ($p>0.05$) on the survival of adult females (Table XXXVI). Cumulative survival by exposure group is displayed in Figure XVI.

3.1.3 Juvenile Males

Overall comparisons indicated a significant difference in the Log Rank test of equality ($p=0.44$, Table XXXVII). Pairwise comparison revealed significant differences in survival between the high and low exposure groups ($p=0.003$). No significant difference in survival exists between the control group and low level or high level exposure groups ($p=0.766$ and $p=0.169$ respectively, Table XXXVIII). Cumulative survival by exposure group is displayed in Figure XVII.

3.1.4 Juvenile Females

Overall comparisons indicated a significant difference in all tests of equality ($p<0.001$, Table XXXIX). Pairwise comparison revealed significant differences in survival between both the control and high exposure ($p=0.006$) and low exposure groups ($p=0.01$). Significant differences in survival also exists between the low and high level exposure groups ($p<0.001$, Table XL). Cumulative survival by exposure group is displayed in Figure XVIII.

3.2 Growth

Growth was measured as weekly mass and recorded in grams.

3.2.1 Adult Males

Exposure to NP did not show a significant impact ($p>0.05$) on the growth of adult males. Time exposed to NP did show a significant effect on growth ($p<0.001$, Table XLI)

3.2.2 Adult Females

The interaction between exposure group and week has a significant impact on growth of adult females ($p=0.008$, Table XLII). The high exposure group is significantly lower than low level exposure group in weeks two, three, four, five, six, eight, and ten ($p<0.05$, Table XLIII). The low exposure group is significantly greater than the control in week two ($p=0.029$). Means and 95% confidence intervals are recorded in Table XLIV.

3.2.3 Juvenile Males

Exposure did not show a significant impact ($p>0.05$) on the growth of juvenile males. Week did show a significant effect on growth ($p<0.001$, Table XLV).

3.2.4 Juvenile Females

Exposure did not show a significant impact ($p>0.05$) on the growth of juvenile females. Week did show a significant effect on growth ($p<0.001$, Table XLVI).

3.3 Gonad Mass

3.3.1 Adult Males

Exposure did not show a significant impact ($p>0.05$) on gonad mass of adult males (Table XLVII).

3.3.2 Adult Females

Exposure did not show a significant impact ($p>0.05$) on gonad mass of adult females (Table XLVIII).

3.3.3 Juvenile Males

Exposure did not show a significant impact ($p>0.05$) on gonad mass of juvenile males (Table XLIX).

3.3.4 Juvenile Females

Exposure did not show a significant impact ($p>0.05$) on gonad mass of juvenile females (Table L).

3.4 Molting

3.4.1. Adult Males

Adult males at low level exposure molted more than controls, while high level exposed males molted the least. Adult males molted the least compared to adult females, juvenile males, and juvenile females (Table

XIX). Adult males had the highest percentages of molts resulting in deaths. The exposed groups had higher rates of deaths after a molting event relative to controls with the low level exposure group having the highest molt related death rate at 88% of molts resulting in death (Figure XX). Low level exposure group had the highest molt related death rates relative to high level exposure and control groups (35% of total deaths). High level exposure group and control group did not have different molt related death rates from each other. The low level exposed adult males had the highest percentage of deaths related to a molting event of any other group (35% of total deaths; Figure XXI).

3.4.2 Adult Females

Adult females showed a dose dependent decrease in total number of molts with the control group having the highest number of molts, followed by the low level exposure group, then high level exposure (Figure XIX). Upon molting, 50% of low level exposed molting adult females died relative to 40% of controls. The high level exposed group had the lowest molt related mortality of adult females (Figure XX). Adult females molted more than adult males but had less molt related deaths. The percent of deaths related to a molting event followed the same dose dependent pattern for number of molts, control with the highest percentage of deaths from molting, followed by low level, then high level (Figure XXI).

3.4.3 Juvenile Males

Juvenile males at low level exposure molted more than controls, while high level exposed males molted the least. Juvenile males molted more than adult males (Table XIX). The exposed groups had higher rates of deaths after a molting event relative to controls with the low level exposure group having the highest death rates when molting of juvenile males (Figure XX). Low level exposure group had the highest molt related death rates relative to high level exposure and control groups (20% of total deaths). The high level exposure group had higher molt related death rates than the control group (Figure XXI).

3.4.4 Juvenile Females

Juvenile female controls had the highest number of molts, followed by high level exposure, then the low level exposure group. Juvenile females molted more than adult females (Figure XIX). Upon molting, low level exposed molting juvenile females died more relative to controls. The high level exposed group had the lowest molt related mortality of all groups (Figure XX). High level exposed juvenile females molted more than juvenile males but had less molt related deaths. The percent of deaths related to a molting event were equal in the control and low level groups with no deaths in the high level group (Figure XXI).

Chapter 5: Discussion

Part I: Behavior

Juvenile males exposed to both low and high-level sub-lethal nonylphenol concentrations spent significantly less time in the correct arm relative to controls. This signifies nonylphenol exposed juvenile males had a decreased ability to find a mate as they spent less time in the arm with the scent of females than the control crayfish. The exposed groups may not have spent as much time in the correct arm due to impaired chemoreceptive ability, developmental delays, or altered endocrine signaling systems. Spending less time in the arm containing female scent implies the exposed groups are not actively searching for a mate or unable to detect one, suggesting impairment in reproductive behavior and/or reduced sexual maturity. The other three groups (adult male, adult female and juvenile female) did not show any clear dose dependent differences in percent of time spent in the correct arm. These results could be attributed to the interaction of age and sex, where the increased susceptibility to toxins and hormones early on in development could make juvenile males more vulnerable to NP's effects on behaviors that come with sexual maturity, impairing them prior to their full development. Nonylphenol's differential effects on juvenile male crayfish could be attributed to the estrogen mimicking quality, affecting females less than males. Changes in reproductive behavior of fish after pesticide exposure during embryonic stages supports these developmental dependent findings (Weis, 2014). Similarly, fathead minnow male larvae exposed to nonylphenol ethoxylates had reduced capacity in competing for spawning sites, while 0.5µg/L of 4-nonylphenol caused impaired social interactions of juvenile killifish (Weis, 2014). Not only did nonylphenol exposed juvenile males spend less time in the correct arm,

they spent significantly less time in an arm and consequently more time in the neutral base during exposure. Spending less time in the arms of the maze means these crayfish spend less time searching for a mate. While the controls and low-level exposed groups spend at least 50% of the time searching the maze every week, the high-level exposed crayfish spend the majority of the trial in the neutral base after one week of exposure. The high-level exposed group shows significant differences between week one of exposure and week eight, while the other groups do not significantly change over time. The majority of time spent in the neutral base in week one by high-level exposed juvenile males suggests a delay in the reproductive behavior of searching for a mate that comes with sexual maturity.

Significant effects of exposure over time on the percentage of time spent in the correct arm and amount of time spent searching were observed in adult females. The interaction of exposure group over time was significant but no specific differences could be identified in the subsequent analysis of percent of time correct. These differences could be attributed to the need of both visual and chemoreceptive cues necessary for female crayfish in the reproductive process, as only chemical cues were provided in this experiment (Breithaupt, 2011).The lowlevel exposure group spent significantly less time searching than other groups in week two. This was also less time than the group spent searching in week one, implying a diminished ability over time. By week four of exposure, the low-level group spends significantly more time searching than in week two and shows no difference relative to the other exposure groups. This change could indicate recovery or adaption to chemical exposure. While the high-level did not show the same disruptive effects as the low-level exposure, this could point towards disruption of endocrine systems rather than other previously suggested mechanisms. Low-level exposure

levels could suppress or activate normal hormonal processes that otherwise would be unaffected by high hormone concentrations. In human uterine cells, low level concentrations of a selective estrogen receptor modulator were shown to inhibit growth while high levels could be stimulatory (Liu et al., 2007). Endocrine disrupting chemicals have been shown to induce different responses dose dependently to varying tissues. Typically, low concentrations of estrogens inhibit transcription while higher levels activate it, low concentrations of endocrine disruptors, such as BPA and NP, were shown to activate genes at concentrations around 0.1nM. Genes are differentially transcribed due to the genes varying estrogen sensitivity. Highsensitivity estrogen responsive genes are activated at these biologically low concentrations. This explains how a single chemical can cause biologically varying responses to changes in concentration (Shioda et al., 2013). The high exposure could also mimic natural processes of the adult female crayfish, explaining the lack of behavior differences among high level exposed and control groups.

The amount of time searching for a mate as a measure of reproductive behavior was significantly affected by developmental stage tested. Adults spent significantly more time searching both arms than juveniles, indicating behaviors necessary for reproduction come with age. This was reiterated in analysis of the juvenile female behavior. In week one, juvenile females spent significantly less time in an arm and more time in the neutral base than in the subsequent weeks, thus demonstrating changes in reproductive behavior over time. This supports the classification of these groups as juveniles, as they did not demonstrate equal time searching as adult reference groups.

These changes in mobility after nonylphenol exposure contributes to decreased reproductive behavior, parallel to decreases in social interactions and mating behavior in several fish species suggests an underlying mechanism impairing aquatic organisms (Weis, 2014). In addition, nonylphenol has been linked to many physiological problems ranging from reproductive development, irregular heartbeats, and loss of normal movements (Cox, 1996; Moore and Waring, 1996; Liney et al. 2006). The differential effects among the four groups shows differences based on developmental stage of exposure, which could lead to future generations of crayfish displaying delayed and impaired reproductive behavior. Without demonstrating normal reproductive behavior, population numbers of crayfish may begin to decline in regions where nonylphenol levels persist, such as areas of agricultural runoff and urban waste water treatment efflux.

Part II: Physiology

The antennules of the crayfish are the primary olfactory organs that are spatially separated, consisting of two lateral and two medial rami. In aquatic environments, crayfish antennules are constantly taking in olfactory information from their surroundings via chemical cues. The antennules can be used for distant food odors, sex discrimination, and agonistic and social behaviors of decapod crustaceans (Corotto et al., 1999; Giri & Dunham, 2000; Moore and Bergman, 2005). Additionally, a recent study demonstrated that the antennules also sample hydrodynamic information (Monteclaro et al., 2010). Both males and females showed dose dependent alterations in chemoreceptive capability after nonylphenol exposure. Control groups showed a significant increase in responsiveness to the water application stimulus compared to when at rest (not stimulated), and the highest response values in the presence of food odors.

The increased responses observed for food stimuli indicates neuron sensitivity to chemical odors specific to food. These high responsive values indicate that more neurons are responding to food than observed in response to the resting condition. Changes in neural responsiveness were observed at both low and high exposure levels. The low-level groups did not show any significant differences in water and food stimuli, which indicates reduced olfactory responsiveness, likely due to less neurons reaching threshold. High-level exposed crayfish had lower response values for food stimuli then water and resting stimuli.

This decreased neural responsiveness could indicate an inhibitory mechanism in response to odor after nonylphenol exposure. Regardless of sex, high-level exposed crayfish showed lower responsive values for food than low-level exposed crayfish, and both were lower than controls. Similar results have been found previously in vertebrate fish after exposure to nonylphenol and other pesticides (Saucier et al., 1991; Hara, 1992; Moore and Waring, 1996; Scholz et al., 2000; Olsen, 2014). Reductions in olfactory electrophysiological responses indicate decreased chemoreceptive sensitivity, which would lead to decreases in behavior requiring olfaction such as reproduction and finding food. This could be caused by blocked chemoreceptors, changes in ion transport and subsequent neuron depolarization, or contaminants traveling to the brain causing subsequent neural damage (Weis, 2014). Moreover, nonylphenol is often used for its adherence property to effectively attach pesticides to plant surfaces (Gutiérrez-Miceli, et al., 2008). If nonylphenol adheres to the antennules and blocks the receptors from receiving olfactory signals necessary to find food then responses would diminish. Therefore, any obstruction of the olfactory receptors via nonylphenol likely

impedes distance chemoreception for long durations and when living in complex odor landscapes, such impedance can be hazardous to survival (Moore and Crimaldi, 2004). The continuous exposure of antennules to odorants also makes them vulnerable to aquatic pollutants. Such pollutants could alter olfactory-mediated behaviors by masking odors, inhibiting detection via chemoreceptors, or changing the behavioral response to a chemical signal (Steele et al., 1992; Klaprat et al., 1992). Whereas fish have a greater potential to recover from damage to the olfactory receptor cells due to high regenerative rates, crayfish only replace the peripheral olfactory receptor cells during the molting process (Zeni et al., 1995; Sandeman and Sandeman, 1996; Harrison et al., 2001). Additionally, the profile of cytochrome P450 or other enzymes capable of metabolizing toxic substances in crayfish olfactory cells remains unclear (James and Boyle, 1998). While pollutant metabolism is well characterized in the mammalian olfactory, little work has been done how aquatic pollutants alter crayfish olfaction and mucosa (Reed and De Matteis, 1989).

Part III: Development

Although our laboratory's previous studies determined 0.15µg/L and 0.3 µg/L to be below lethal levels for crayfish, both juvenile male and female survival in this study were affected by sub-lethal nonylphenol concentrations. Juvenile males exposed to high NP concentrations had lower survival than low level exposed juvenile males. While neither showed differences from the control group, it still demonstrates a NP dose dependent change in survival below current EPA levels. Juvenile females exposed to high NP concentrations had lower survival than both control and low level groups. This indicates that concentrations

previously determined sub-lethal were likely measured using adult subjects. While undergoing developmental changes, increased rates of cell division, and tissue maturation, juveniles could be more vulnerable to even relatively low concentrations of contaminants. Similarly, fish embryos exposed to nonylphenol showed decreased survival and diminished development (Soares, 2008). Although the 0.3 µg/L exposure used in this experiment is well below the chronic EPA guidelines and indefinite exposure in Canada, it still negatively impacts survival in juvenile crayfish. In doing so, less crayfish will survive to reproductive age, leading to limited numbers of crayfish to procreate.

Adult females were the only group in which exposure over time affected growth. The high-level exposure group had diminished growth relative to the low-level for several weeks. The low-level group had increased growth relative to controls, however, this only occurred in week two. The return of low-level exposed crayfish to growth similar to that of the controls could be attributed to the adult females adapting to the continuous low-level exposure. Lowlevel NP concentrations have also been found to enhance growth and reproduction in nematodes (Soares, 2008). The high-level, having the opposite effect on growth as the lowlevel, could indicate a stimulatory/inhibitory relationship dependent on concentration, similar to the concentration dependent effects of estrogen in human females. If a similar effect exists in crustaceans, it would explain why adult females were affected while the other groups were not.

The decreased growth rate in high-level exposed females could help explain the decreased total number of molting events of high-level exposed adult females relative to controls. As molting typically occurs in response to organism growth, diminished growth would

lead to decreased molting events. Less molting events due to less growth would lead to proportionate decreases in molt related deaths as seen among high level exposed females. Previous research has demonstrated that significant reductions in growth resulted when marine mysid shrimp were exposed to sub-lethal concentrations of nonylphenol, along with a decrease in the number of molts (Hirano et al., 2009). The exposed adult male groups had higher rates of deaths after a molting event relative to controls with the low-level exposure group having the highest molt related death rate at 88% of molts resulting in death suggesting a disruption of molting, leading to increased mortality. The small increase in molts of adult males exposed to low-level NP concentrations relative to controls paired with the high percentage of molts resulting in death suggests an impairment in the molting mechanism. This could be attributed to inappropriate stimulation of molting events when crayfish are not physiologically ready to molt by normal growth standards or diminished recovery from molting events. Typically, crayfish molt when epithelial Y-organs release ecdysone are activated and neuroendocrine Xorgans that release molt inhibiting hormone are inhibited (Longshaw, 2016). If nonylphenol disrupts either of these hormone related processes, changes in molting frequency would exist in exposed organisms as seen among different exposure groups. A direct correlation of alkylphenol concentration and juvenile hormone activity has been documented in lobsters, this increased activity may inhibit shell hardening after molting, consequently increasing vulnerability to disease or predators (Biggers and Laufer, 2004). This could likely be occurring in crayfish, leading to increased mortality related to molting events and the high proportion of adult male deaths related to molting events in the low level exposed groups. The decreased number of molts in the high-level exposed adult males paired with no differences in growth

between groups could imply an inhibition of molting due to increased NP concentrations. Lower molting rates explain the relatively low percentage of high-level exposed adult male deaths due to molting. The juveniles molting at increased rates relative to adult groups is explained by increased growth rates due to developmental stage. Much like in adult females, juvenile females exposed to NP showed decreased molt numbers relative to controls. Juvenile males follow a similar pattern to adult males, highest molt numbers among the low-level groups and lowest molting numbers in the high level exposed juvenile males. If inappropriately activating the endocrine signals for molting, an increased percentage of deaths would be related to molting, as observed in both male groups.

General Summary

High-level nonylphenol exposed crayfish molted less than controls in all groups. As molting is triggered by ecdysone release from the Y-organ or inhibited by molt inhibiting hormone (MIH) release from the X-organ, this decrease in molting could be attributed to interference with either aspect of this endocrine controlled system.

Increases in MIH or decreases in ecdysone are potential mechanisms for delayed or diminished molting. However, changes in the concentrations of these hormones are not the only possible site of interference as competitive receptor binding inhibition could change molting frequency. Similar to our results, other xenoestrogens have been found to interfere with the molting process of crabs. These endocrine disrupting molecules may be blocking ecdysone receptors in the epidermal cuticle and in the X-organ, ultimately leading to delayed or diminished molting (Zou and Fingerman, 1999). Estrogen mimics such as BPA were also found

to antagonize ecdysone receptors in crustaceans at sub-lethal concentrations (Dinan et al., 2001). By antagonizing these receptors, the effects of ecdysone would decrease, consequently causing molting to decrease.

While interfering with ecdysone receptors has been observed after exposure to estrogen mimicking molecules, changes in the sensitivity of either the X or Y-organs has also been shown to affect molting. Molting associated with increased ecdysone release is correlated with not only decreases in MIH but stage dependent changes in sensitivity of the Y-organ (Nakatsuji, 2009). Increased Y-organ sensitivity would lead to increases in ecdysone concentration while increased X-organ sensitivity would lead to increased MIH. It has been suggested that estrogens act on ecdysteroid receptors and decrease their sensitivity by increasing the threshold of ecdysone needed to trigger molting (Andersen et al., 2001). Estrogens and estrogen mimics inhibiting and decreasing sensitivity of ecdysone receptors parallels the effects of naturally occurring MIH. The diminished ecdysone concentrations and decreased receptor activity could suggest these estrogen mimics also mimic MIH. Since crustaceans do not possess classic estrogen receptors like those found in mammals, another mechanism for endocrine disruption likely exists in invertebrates. Estrogen mimics are most commonly linked to increased feminization marked by increased vitellogenisis (Jones et al., 2000; Chen, 2016). MIH was also found to induce vitellogenesis in crabs (Zmora et al., 2009). Wastewater treatment efflux containing xenoestrogens increased expression of CYP enzymes and HSP70 (Xu et al., 2015; Chen, 2016). Similarly, increases in CYP enzymes and HSP70 correlated with decreases in ecdysone were observed in lobsters exposed to estrogen which was associated with their delayed molting (Snyder and Mulder, 2001). Similar receptor

interactions between nonylphenol and MIH receptors would demonstrate a mimicking relationship. While the MIH receptor has not been characterized, recent research suggests that three amino acid residues (serine, isoleucine, and asparagine) belong to the MIH structure region interacting with the MIH receptor, as these are crucial for functional activity (Katayama et al., 2004). The polar region of NP could mimic asparagine while the nonpolar carbon chain could resemble isoleucine and serine. The similar effects and structure exhibited by nonylphenol and MIH would identify a mechanism of action for NP in crustaceans.

Reduced molting leads to reduced olfactory cell turnover. Crayfish only replace the peripheral olfactory receptor cells during the molting process (Zeni et al., 1995; Sandeman and Sandeman, 1996; Harrison et al., 2001). As the high level exposed groups exhibited diminished olfactory responsiveness to food stimuli, the decreased molting rates would lead to extended periods of olfactory impairment as crayfish would be unable to regenerate new olfactory receptor cells. The groups exhibiting changes in molting due to NP exposure would also likely exhibit behavioral changes. The juvenile male crayfish exposed to the high-level NP concentrations exhibited delays in reproductive behavior, spent less time in the correct arm ,and less time searching for a mate relative to controls and simultaneously had depressed molting rates relative to controls. This diminished reproductive behavior is likely due to the decrease in molting and subsequent regeneration of olfactory cells. Similarly, copepods exposed to estrogens during juvenile stages showed delayed development and decreased sensitivity to ecdysone, leading to decreased molting (Andersen et al., 2001). Like the exposed juvenile males, adult females also had diminished reproductive behavior correlated with decreased molting. Low-level exposed adult females in week two spent less time searching and were

growing more relative to controls. Despite their increased growth, they did not have increases in molting. The delay in molting would explain the decreased searching, as their chemoreceptive capacity would be hindered due to prolonged NP exposure as revealed in the electrophysiological recordings. Albeit delayed, the adult females restored searching capacity in week four would be explained by the regeneration of the olfactory receptive cells from molting. In adult males exposed to high-level NP, the decrease in molting rate relative to controls are the least pronounced of all the groups. The small variability between high-level and control molting rates paired with no significant differences in behavior of adult males further suggests the close relationship between molting frequency and behavior.

Conclusion

The endocrine disrupting effects of nonylphenol alters reproductive behavior through chronically hindering electrophysiology and altering developmental processes. Due to the crayfishes' role in the food web, they are critical to a healthy ecosystem and exposure to chemicals like nonylphenol will likely have resonating effects within and beyond the crayfish population. Despite these concentrations being below current regulations, detrimental effects still exist. This necessitates the re-evaluation and implementation of an indefinite exposure, low-level guideline if nonylphenol continues to be used in a wide variety of industries.

Appendix: Tables and Figures

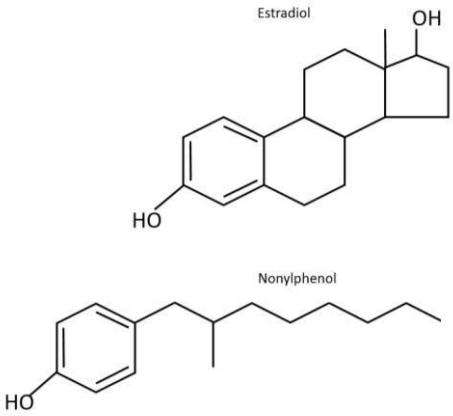

Fig. I. Chemical structures of estradiol and nonylphenol

Experiment I: Behavior

Table I.

Percent of Time Correct: Adult Male

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure Group	2	36.150	.126	.882
Week	7	195.811	1.535	.157
Exposure Group x Week	14	195.256	1.523	.106

a. Dependent Variable: % Correct.

Table II.

Percent of Time Correct: Adult Female

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure Group	2	36.537	1.767	.185
Week	7	206.049	.476	.851
Exposure Group x Week	14	205.498	1.871	**.031***

a. Dependent Variable: % Correct.
* Indicates significant interaction

Table III.

Percent of Time Correct: Juvenile Male

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure Group	2	118.004	5.235	**.007***
Week	7	198.791	1.170	.322
Exposure Group x Week	13	187.165	.948	.505

a. Dependent Variable: % Correct.

* Indicates significant interaction

Table IV.

Percent of Time Correct: Juvenile Male

Pairwise Comparisons[a]

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
						Lower Bound	Upper Bound
Low	High	.422	2.666	133.675	1.000	-6.041	6.886
	Control	-7.126*	2.632	126.911	**.023***	-13.511	-.741
High	Low	-.422	2.666	133.675	1.000	-6.886	6.041
	Control	-7.548*	2.525	112.334	**.010***	-13.685	-1.412
Control	Low	7.126*	2.632	126.911	**.023***	.741	13.511
	High	7.548*	2.525	112.334	**.010***	1.412	13.685

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: % Correct.

c. Adjustment for multiple comparisons: Bonferroni.

Table V.

Percent of Time Correct: Juvenile Male

Estimates[a]

Group	Mean	Std. Error	df	95% Confidence Interval	
				Lower Bound	Upper Bound
Low	26.651	1.954	146.836	22.789	30.514
High	26.229	1.813	118.885	22.640	29.818
Control	33.777	1.759	106.931	30.289	37.265

a. Dependent Variable: % Correct.

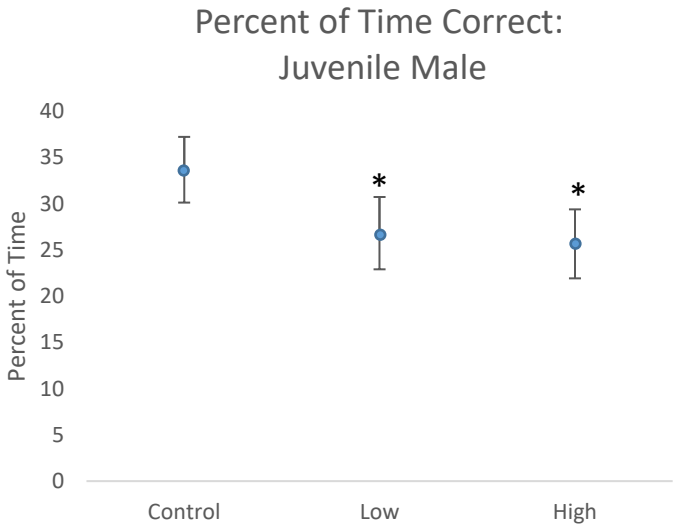

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Fig. II. Percent of Time Correct: Juvenile Males. The percent of time each exposure group spent in the correct arm during a trial. Error bars represent 95% confidence interval. Low-level and high-level exposure groups spent significantly less time on the correct side than controls.

*. The mean difference is significant at the .05 level

Table VI.

Percent of Time Correct: Juvenile Female

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure Group	2	114.998	.328	.721
Week	6	146.604	.811	.563
Exposure Group x Week	12	147.050	.769	.681

a. Dependent Variable: % Correct.

Table VII.

Percent of Time Correct: Comparison by Sex

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Sex	1	380.610	2.321	.128

a. Dependent Variable: % Correct.

Table VIII.

Percent of Time Correct: Comparison by Age

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Age	1	401.891	1.322	.251
Exposure Group	2	426.274	4.082	**.018***
Week	7	709.353	1.234	.281

a. Dependent Variable: % Correct.

*. The mean difference is significant at the .05 level.

Table IX.

Percent of Time Correct: Comparison by Age

Pairwise Comparisons[a]

Age	(I) Exposure	(J) Exposure	Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
Adult	High	Low	-1.661	1.259	437.414	.563	-4.685	1.364
		Control	-3.501*	1.226	408.321	**.014***	-6.448	-.554
	Control	Low	1.840	1.256	434.989	.431	-1.178	4.859
		High	3.501*	1.226	408.321	**.014***	.554	6.448
Juvenile	High	Low	-1.661	1.259	437.414	.563	-4.685	1.364
		Control	-3.501*	1.226	408.321	**.014***	-6.448	-.554
	Control	Low	1.840	1.256	434.989	.431	-1.178	4.859
		High	3.501*	1.226	408.321	**.014***	.554	6.448

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: % Correct.

c. Adjustment for multiple comparisons: Bonferroni.

Table X.

Percent of Time Correct: Comparison by Age

Estimates[a]

Age	Exposure	Mean	Std. Error	df	95% Confidence Interval	
					Lower Bound	Upper Bound
Adult	Low	29.664	.987	433.932	27.724	31.604
	High	28.003	.937	399.217	26.161	29.844
	Control	31.504	.947	400.601	29.642	33.366
Juvenile	Low	28.023	1.397	452.449	25.278	30.768
	High	26.362	1.391	439.047	23.628	29.096
	Control	29.864	1.363	426.141	27.184	32.543

a. Dependent Variable: % Correct.

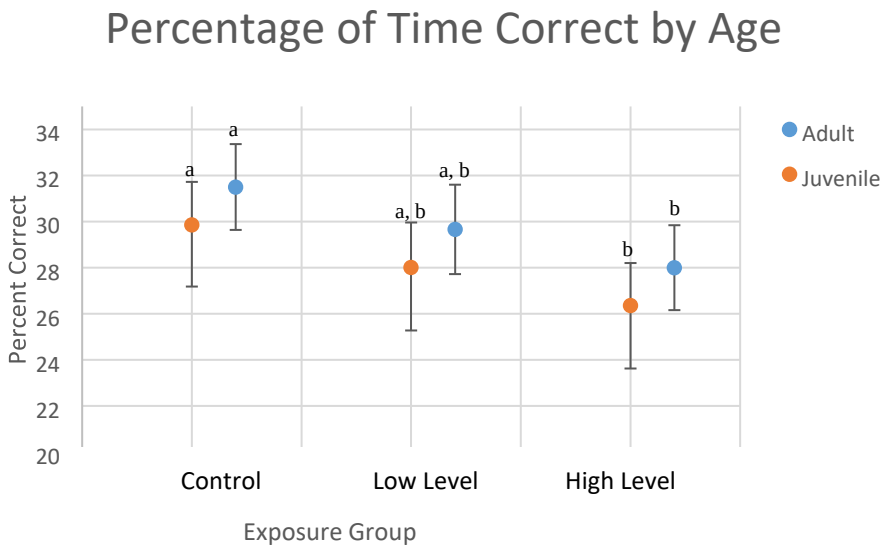

Fig. III. Percent of Time Correct by Age. The percent of time adults and juveniles of each exposure group spent in the correct arm during a trial. Error bars represent 95% confidence interval. Controls spent significantly more time on the correct side than high-level exposure groups.

[a] and [b] Indicate the mean difference is significant at the .05 level

Table XI.

Percent of Time Choosing: Adult Male

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure Group	2	198.934	.551	.577
Week	7	48.027	1.321	.261
Exposure Group x Week	14	48.469	1.287	.250

a. Dependent Variable: % of time choosing a side.

Table XII.

Percent of Time Choosing: Adult Female

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure Group	2	155.728	2.474	.088
Week	7	47.068	1.296	.273
Exposure Group x Week	14	47.080	1.926	**.048***

a. Dependent Variable: % Correct.

* Indicates significant interaction

Table XIII.

Percent of Time Choosing: Adult Female

Pairwise Comparisons[a]

Week	(I) Group	(J) Group	Mean Difference (I-J)	Sig.[c]	95% Confidence Interval for Difference[c]	
					Lower Bound	Upper Bound
2	Low	High	-13.738*	**.050***	-27.461	-.014
		Control	-22.109*	**.001***	-35.833	-8.385
	High	Low	13.738*	**.050***	.014	27.461
		Control	-8.372	.400	-22.095	5.352
	Control	Low	22.109*	**.001***	8.385	35.833
		High	8.372	.400	-5.352	22.095

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: % of time choosing a side.

c. Adjustment for multiple comparisons: Bonferroni

Table XIV.

Percent of Time Choosing: Adult Female

Estimates[a]

Group	Week	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Low	1	66.509	59.944	73.075
	2	45.832	38.004	53.659
	3	59.283	53.137	65.428
	4	63.318	57.518	69.118
	5	57.382	46.992	67.772
	6	56.261	50.793	61.730
	7	60.739	55.506	65.972
	8	60.264	47.745	72.784
Group 3 High	1	53.879	45.838	61.920
	2	59.569	51.741	67.397
	3	63.697	57.551	69.843
	4	59.169	53.874	64.464
	5	55.002	45.517	64.486
	6	55.916	50.728	61.104
	7	59.351	54.671	64.031
	8	60.818	50.344	71.292
Group_1 Control	1	61.765	54.573	68.957
	2	67.941	60.113	75.769
	3	61.026	54.880	67.172
	4	66.771	61.476	72.066
	5	58.526	49.041	68.010
	6	61.460	56.514	66.406
	7	58.725	54.263	63.188
	8	60.219	49.178	71.260

a. Dependent Variable: % of time choosing a side.

Table XV.

Percent of Time Choosing: Adult Female

Pairwise Comparisons by Week[a]

Group	(I) Week	(J) Week	Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
Low	1	2	20.678*	5.004	59.593	**.003***	4.310	37.045
	2	4	-17.486*	4.784	59.882	**.015***	-33.131	-1.841

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: % of time choosing a side.

c. Adjustment for multiple comparisons: Bonferroni.

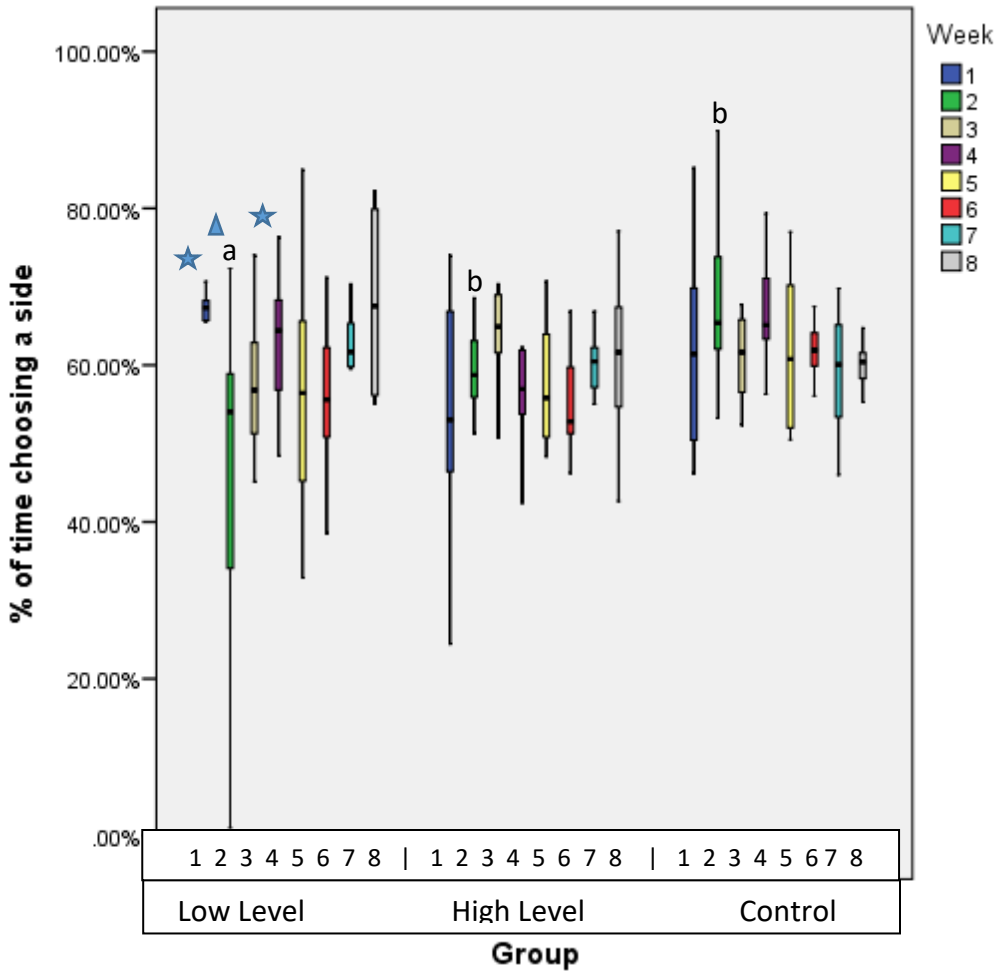

Fig. IV. Percent of Time Choosing an Arm by Week: Adult Female. The percent of time adult females of each exposure group spent choosing an arm rather than the neutral base during a trial each week.

[a] and [b] Indicate the mean difference is significant at the .05 level

★ and ▲ are significantly different at the .05 level

Table XVI.

Percent of Time Choosing: Juvenile Male

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure Group	2	182.125	2.866	.060
Week	7	53.320	3.025	**.009***
Exposure x Week	13	55.809	4.945	**.000***

a. Dependent Variable: % of time choosing a side.

*. The mean difference is significant at the .05 level.

Table XVII.

Percent of Time Choosing: Juvenile Male

Pairwise Comparisons[a]

Week	(I) Exposure	(J) Exposure	Mean Difference (I-J)	Std. Error	df	Sig.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
6	Low	Control	-17.155*	5.248	31	**.008***	-30.436	-3.874

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: % of time choosing a side.

b. Adjustment for multiple comparisons: Bonferroni.

Table XVIII.

Percent of Time Choosing: Juvenile Male

Estimates[a]

Exposure Group	Week	Mean	Std. Error	df	95% Confidence Interval	
					Lower Bound	Upper Bound
Low	1	49.626	6.669	33	36.059	63.193
	2	56.102	3.859	31	48.232	63.971
	3	61.241	3.615	33.000	53.887	68.595
	5	62.713	4.905	31.000	52.709	72.717
	6	52.278	4.003	33.000	44.133	60.423
	7	62.278	3.785	31.000	54.559	69.998
	8	54.288	3.326	29.000	47.486	61.090
High	1	31.638	6.669	33	18.070	45.205
	2	50.601	4.227	31	41.980	59.222
	3	50.334	3.615	33.000	42.980	57.688
	4	70.676	2.204	21	66.093	75.260
	5	55.122	4.478	31.000	45.989	64.254
	6	57.614	4.003	33.000	49.469	65.759
	7	51.911	4.146	31.000	43.455	60.367
	8	63.097	3.643	29.000	55.646	70.548
Control	1	51.199	6.669	33	37.632	64.766
	2	62.026	3.859	31	54.156	69.895
	3	60.298	3.615	33.000	52.943	67.652
	4	53.268	2.110	21	48.880	57.657
	5	57.251	4.478	31.000	48.119	66.383
	6	69.433	4.003	33.000	61.288	77.578
	7	58.436	3.785	31.000	50.717	66.155
	8	58.053	3.643	29.000	50.602	65.504

a. Dependent Variable: % of time choosing a side.

b. This level combination of factors is not observed, thus the corresponding population marginal mean is not estimable.

Table XIX.

Percent of Time Choosing: Juvenile Male

Pairwise Comparisons by Week[a]

Group	(I) Week	(J) Week	Mean Difference (I-J)	Std. Error	df	Sig.[e]	95% Confidence Interval for Difference[e]	
							Lower Bound	Upper Bound
High	1	4	-39.039*	7.023	39.856	**.000***	-62.554	-15.524
		6	-25.977*	7.778	54.053	**.043***	-51.539	-.414
		8	-31.460*	7.599	50.518	**.004***	-56.524	-6.395
	2	4	-20.075*	4.767	45.215	**.003***	-35.902	-4.249
	3	4	-20.342*	4.234	51.018	**.000***	-34.299	-6.385
	4	7	18.765*	4.696	45.619	**.007***	3.184	34.346
Control	4	6	-16.165*	4.526	48.055	**.023***	-31.135	-1.195

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: % of time choosing a side.

b. The level combination of factors in (J) is not observed.

c. The level combination of factors in (I) is not observed.

e. Adjustment for multiple comparisons: Bonferroni.

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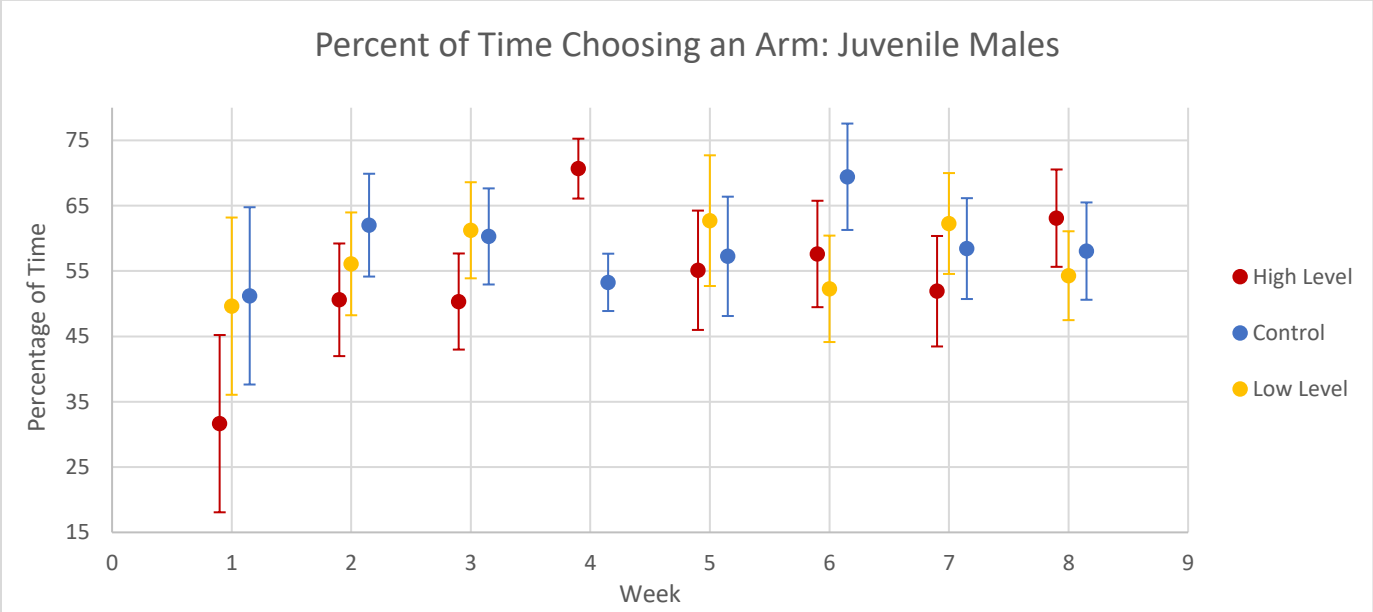

Fig. V. Percent of Time Choosing an Arm by Week: Juvenile Males. The percent of time juvenile males of each exposure group spent in an arm rather than the neutral base during a trial each week. High-level exposed juvenile males during week one spend less than 50% of their time in an arm, the control and low level groups spend at least 50% of the trial exploring arms every week of exposure.

Table xx.

Percent of Time Choosing: Juvenile Female

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure Group	2	146.803	.010	.990
Week	6	54.646	2.589	**.028***
Exposure * Week	12	59.177	.848	.603

a. Dependent Variable: % of time choosing a side.
* Indicates significant interaction

Table XXI.

Percent of Time Choosing: Juvenile Female

Pairwise Comparisons[a]

(I) Week	(J) Week	Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
						Lower Bound	Upper Bound
1	2	-12.507*	4.297	45.791	**.006***	-21.157	-3.856
	3	-10.727*	4.889	61.779	**.032***	-20.502	-.953
	5	-9.115	4.748	58.747	.060	-18.617	.387
	6	-13.768*	4.409	49.209	**.003***	-22.628	-4.909
	7	.320	6.426	64.390	.960	-12.516	13.156
	8	-12.447*	5.014	55.876	**.016***	-22.491	-2.402
2	3	1.780	3.407	57.379	.603	-5.043	8.602
	5	3.392	3.201	60.149	.294	-3.011	9.795
	6	-1.262	2.673	67.965	.638	-6.595	4.072
	7	12.827*	5.385	43.058	**.022***	1.967	23.687
	8	.060	3.581	37.568	.987	-7.191	7.311
3	5	1.612	3.960	68.103	.685	-6.291	9.515
	6	-3.041	3.547	61.958	.395	-10.131	4.049
	7	11.047	5.868	55.682	.065	-.710	22.804
	8	-1.719	4.279	53.315	.689	-10.300	6.861
5	6	-4.653	3.349	64.401	.170	-11.344	2.037
	7	9.435	5.751	52.907	.107	-2.101	20.971
	8	-3.332	4.116	50.059	.422	-11.599	4.936
6	7	14.088*	5.474	45.402	**.013***	3.065	25.112
	8	1.322	3.720	41.523	.724	-6.188	8.832
7	8	-12.767*	5.975	54.641	**.037***	-24.742	-.791

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: % of time choosing a side.

c. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table XXII.

Percent of Time Choosing: Juvenile Female

Estimates[a]

Week	Mean	Std. Error	df	95% Confidence Interval	
				Lower Bound	Upper Bound
1	47.266	3.922	32.971	39.286	55.246
2	59.773	1.757	35.485	56.208	63.338
3	57.993	2.920	34.835	52.065	63.921
5	56.381	2.676	34.325	50.945	61.818
6	61.034	2.014	34.279	56.943	65.126
7	46.946	5.091	34.876	36.611	57.282
8	59.713	3.128	23.077	53.244	66.182

a. Dependent Variable: % of time choosing a side.

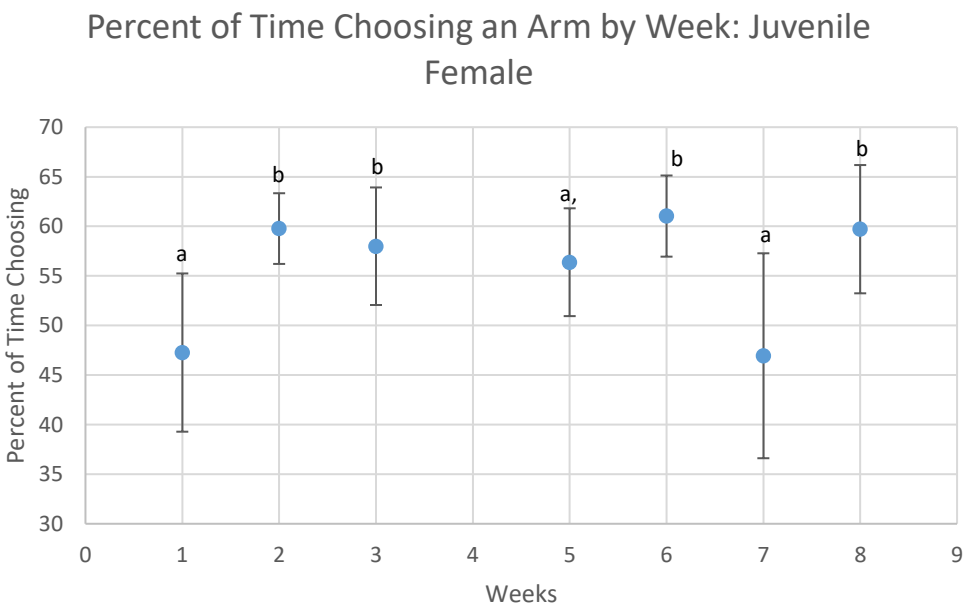

Fig. VI. Percent of Time Choosing an Arm by Week: Juvenile Females. The percent of time juvenile females spent in the correct arm during a trial each week.

[a] and [b] are significantly different at the .05 level

Table XXIII.

Percent of Time Choosing Group Comparison

Group Changes from Week One to Week Eight

Group Tested	Exposure group changing from wk1 to wk8	Sig	Week One: 95% Confidence Interval		Week Eight: 95% Confidence Interval		Does group change over time?
			Lower Bound	Upper Bound	Lower Bound	Upper Bound	
Adult Male	None	.261	-	-	-	-	No
Adult Female	None	.273	-	-	-	-	No
Juvenile Male	High only	**.004***	18.070	45.205	55.646	70.548	Yes, increases
Juvenile Female	All	**.016***	39.286	55.246	53.244	66.182	Yes, increases

*. The mean difference is significant at the .05 level.

Table XXIV.

Percent of Time Choosing: Comparison by Sex

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Sex	1	884.872	3.230	.073

a. Dependent Variable: % of time choosing a side.

Table XXV.

Percent of Time Choosing: Comparison by Age

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Age	1	884.148	6.399	**.012***

a. Dependent Variable: % of time choosing a side.

*. The mean difference is significant at the .05 level.

Table XXVI.

Percent of Time Choosing: Comparison by Age

Pairwise Comparisons[a]

Group	(I) Age	(J) Age	Mean Difference (I-J)	Std. Error	df	Sig.[c]	95% Confidence Interval for Difference[c]	
							Lower Bound	Upper Bound
Low	Adult	Juvenile	3.135*	1.239	884.148	**.012***	.703	5.568
High	Adult	Juvenile	3.135*	1.239	884.148	**.012***	.703	5.568
Control	Adult	Juvenile	3.135*	1.239	884.148	**.012***	.703	5.568

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: % of time choosing a side.

c. Adjustment for multiple comparisons: Bonferroni.

Table XXVII.

Percent of Time Choosing: Comparison by Age

Estimates[a]

Age	Group	Mean	Std. Error	df	95% Confidence Interval	
					Lower Bound	Upper Bound
Adult	Low	58.117	.869	969.390	56.411	59.823
	High	58.249	.831	971.245	56.619	59.879
	Control	60.501	.840	969.722	58.854	62.149
Juvenile	Low	54.981	1.218	941.493	52.591	57.371
	High	55.113	1.210	924.266	52.738	57.489
	Control	57.366	1.191	929.229	55.028	59.704

a. Dependent Variable: % of time choosing a side.

Figure VII.

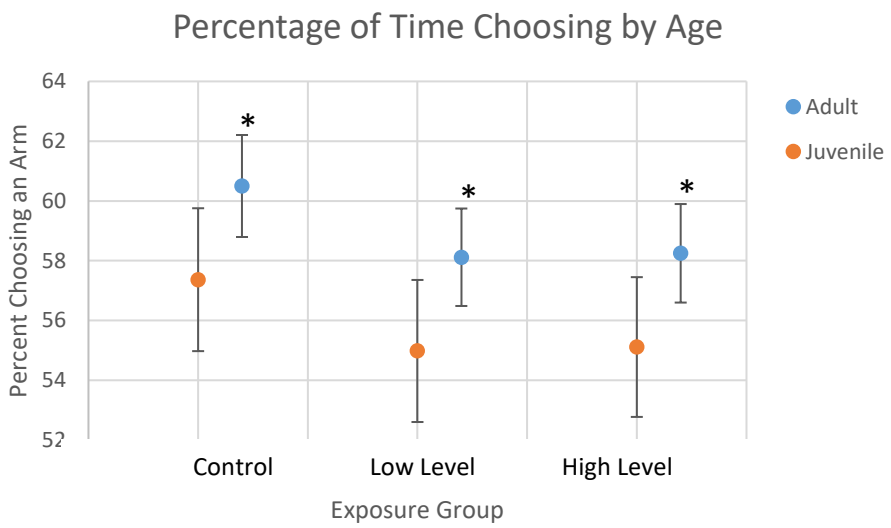

Fig. VII. Percent of Time Choosing by Age. The percent of time adults and juveniles of each exposure group spent choosing an arm rather than the neutral base during a trial each week. Adults spent significantly more time choosing an arm than juveniles

* Indicate the mean difference is significant at the .05 level

Experiment II: Physiology

Table XXVIII.
Electrophysiological Recordings by Exposure Group (Untransformed): Adult Male

Analysis Variable : mV					
Sex	**Stimuli**	**Group**	**Lower Quartile**	**Mean**	**Upper Quartile**
Female	Resting	Control	5.6885293	5.7939631	5.9566214
		Low level	6.6834392	6.8382053	6.9984200
		High Level	6.4195886	6.9133092	7.3282453
	Water	Control	11.4815362	12.2311516	12.5892541
		Low level	57.5439937	59.4768825	60.9536897
		High Level	23.4422882	35.8581382	47.8630092
	Food	Control	81.2830516	91.4939584	98.8553095
		Low level	56.2341325	61.8842482	69.1830971
		High Level	0.5432503	4.3187772	7.8523563
Male	Resting	Control	6.2016492	6.7321295	7.3706739
		Low level	5.6234133	5.6890874	5.7877158
		High Level	6.5691200	7.7886344	8.6099375
	Water	Control	22.1309471	24.0804786	25.7039578
		Low level	23.7137371	23.8845851	23.9883292
		High Lev	27.2270131	44.3557307	60.2559586
	Food	Control	50.7293140	78.5870766	111.5596635
		Low level	22.3872114	23.1159231	24.2661010
		High Lev	0.5623413	4.2397996	7.9432823

Electrophysiological Recordings by Exposure Group: Adult Male

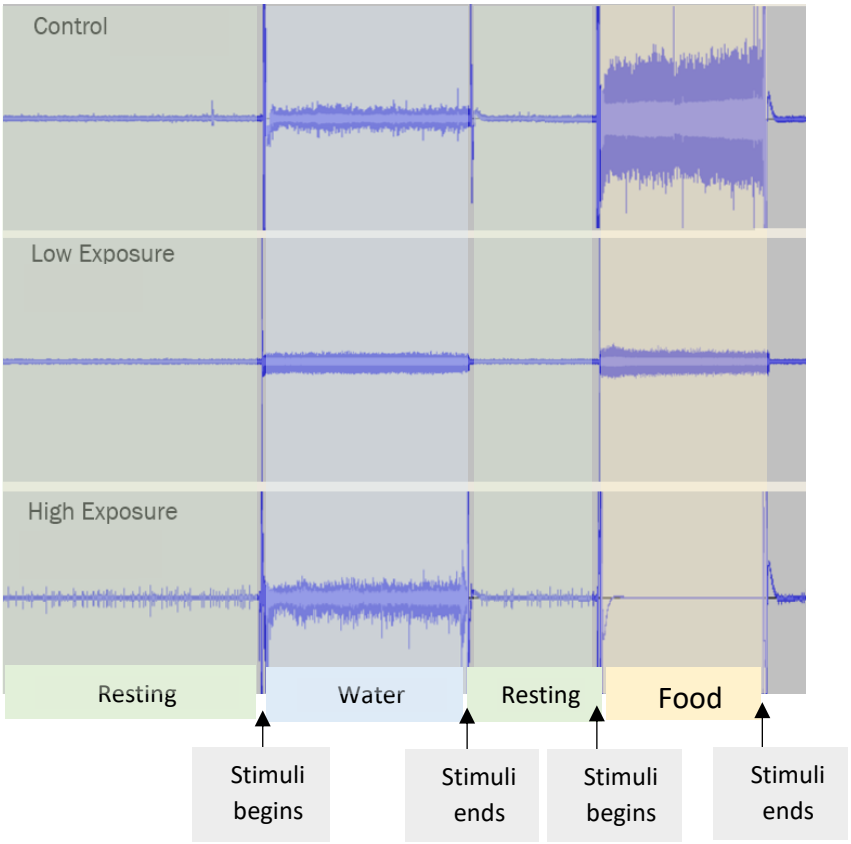

Fig. VIII. Electrophysiological Recordings by Exposure Group: Adult Male. Control (top), Low-Level (middle), and High-level (bottom) raw output of neuron responsiveness to three conditions: resting (green), water stimuli (blue), and food slurry (yellow).

Table XXIX.

Electrophysiological Recordings (SQRT Transformed): Adult Male

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Exposure	2	2	3.26	0.2350
Stimuli	2	4	314.74	**<.0001***
Exposure x Stimuli	4	4	316.14	**<.0001***

*. The mean difference is significant at the .05 level.

Table XXX.

Electrophysiological Recordings (SQRT Transformed): Adult Male

Exposure	Stimuli	Mean	Mode	Std. Deviation	25% Q1	Median	75% Q3
Control	Resting	2.59133376	2.497468	0.13222	2.49030	2.563019	2.71489
	Water	4.8986281	4.731513	0.29269384	4.70435	4.813933	5.06991
	Food	8.65654043	9.067760	1.92426931	7.12139	8.886931	10.56214
Low Level	Resting	2.384838	2.371374	0.04133	2.37137	2.391948	2.40576
	Water	4.886854	4.869675	0.05810	4.86968	4.897788	4.89779
	Food	4.806314	4.813933	0.12505	4.92606	4.813933	4.73151
High Level	Resting	2.781197	2.738420	0.23391	2.56302	2.770129	2.93427
	Water	6.514206	5.217951	1.40275	5.21795	6.607372	7.76247
	Food	1.810789	0.749894	0.98943	0.749894	2.372002	2.818383

Table XXXI.

Electrophysiological Recordings (SQRT Transformed): Adult Male

Pairwise Comparisons [a]

Exposure	(I) Stimuli	Exposure	(J) Stimuli	Mean Difference (I-J)	Sig.[c]
Control	Resting	Control	Water	-2.3204	**0.0002***
			Food	-5.8875	**<.0001***
		Low Level	Resting	0.2065	0.8159
			Water	-2.2955	0.0505
			Food	-2.2150	0.0543
		High Level	Resting	-0.1899	0.7934
			Water	-3.9198	**0.0045***
			Food	0.7805	0.3129
	Water	Control	Food	-3.5671	**<.0001***
		Low Level	Resting	2.5269	**0.0383***
			Water	0.02483	0.9775
			Food	0.1054	0.9041
		High Level	Resting	2.1305	**0.0347***
			Water	-1.5994	0.0782
			Food	3.1009	**0.0101***
	Food	Low Level	Resting	6.0939	**0.0018***
			Water	3.5919	**0.0122***
			Food	3.6725	**0.0109***
		High Level	Resting	5.6976	**0.0011***
			Water	1.9677	**0.0436***
			Food	6.6680	**0.0006***
Low Level	Resting	Low Level	Water	-2.5020	**0.0007***
			Food	-2.4215	**0.0005***
		High Level	Resting	-0.3964	0.6581
			Water	-4.1263	**0.0077***
			Food	0.5740	0.5269
	Water	Low Level	Food	0.08054	0.7465
		High Level	Resting	2.1057	0.0641
			Water	-1.6242	0.1224
			Food	3.0761	**0.0206***
	Food	High Level	Resting	2.0251	0.0693
			Water	-1.7048	0.1070
			Food	2.9955	**0.0217***
High Level	Resting	High Level	Water	-3.7299	**<.0001***
			Food	0.9704	**0.0060***
	Water	High Level	Food	4.7003	**<.0001***

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: SQRTmV.

c. Adjustment for multiple comparisons: Bonferroni

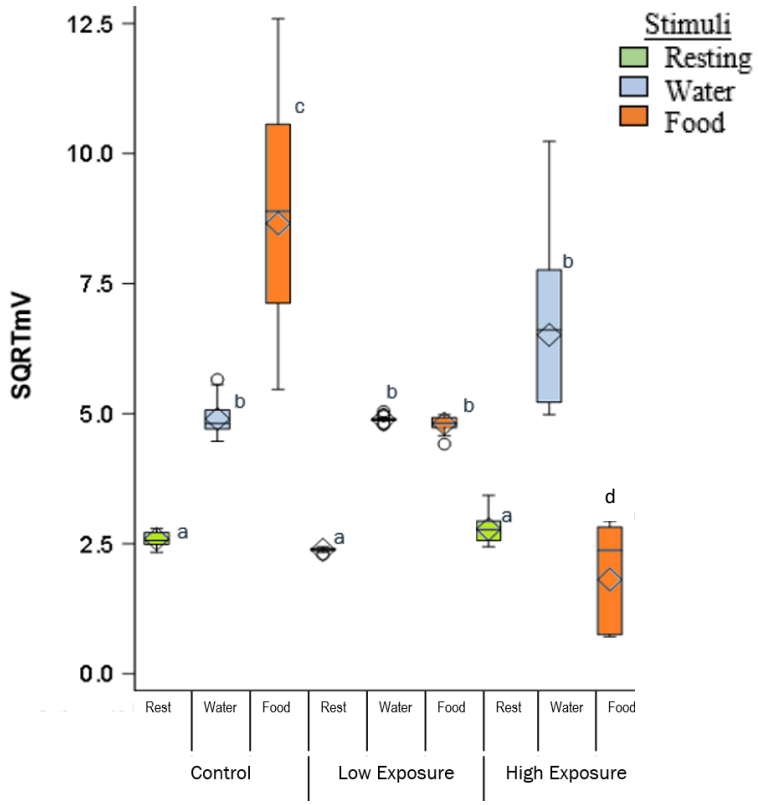

Fig. IX. Electrophysiological Recordings (SQRT Transformed): Adult Male. Neuron responsiveness of each exposure group of adult males to three conditions: resting (green), water stimuli (blue), and food slurry (orange).

[a], [b], [c], [d] are significantly different at the .05 level.

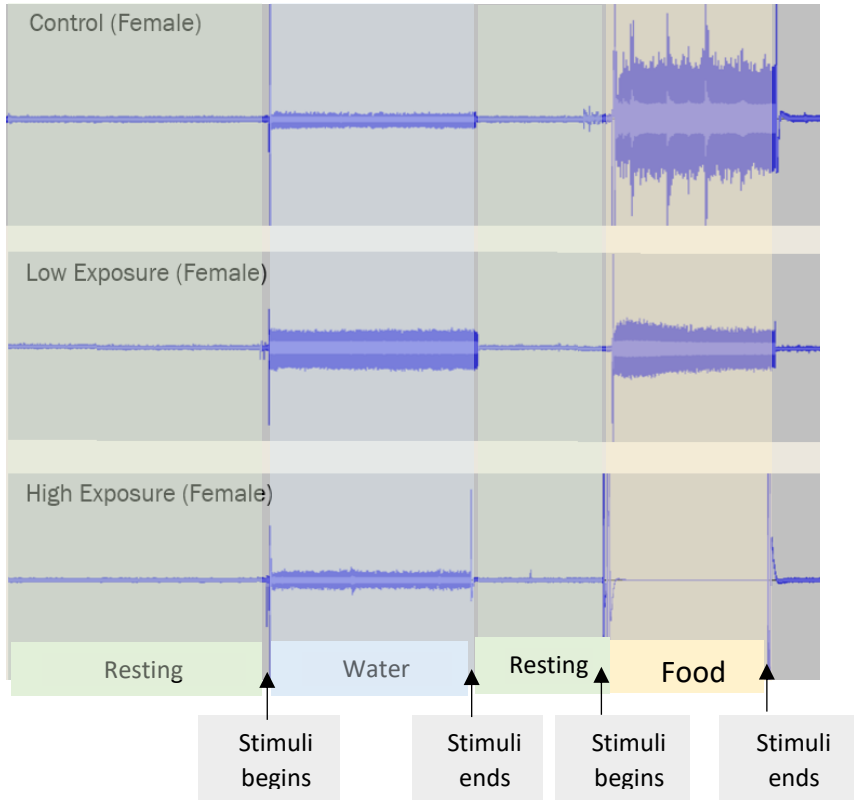

Fig. X. Electrophysiological Recordings by Exposure Group: Adult Female. Control (top), Low-Level (middle), and High-level (bottom) raw output of neuron responsiveness to three conditions: resting (green), water stimuli (blue), and food slurry (yellow).

Table XXXII.

Electrophysiological Recordings (SQRT Transformed): Adult Female

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Exposure	2	1	2.38	0.4169
Stimuli	2	2	2294.75	**0.0004***
Exposure x Stimuli	4	2	1653.97	**0.0006***

*. The mean difference is significant at the .05 level.

Table XXXIII.
Electrophysiological Recordings (SQRT Transformed): Adult Female

Exposure	Stimuli	Mean	Mode	Std Deviation	25% Q1	Median	75% Q3
Control	Resting	2.406697	2.440619	0.04299	2.38506	2.412682	2.44062
	Water	3.49312206	3.408003	0.17487	3.38844	3.408003	3.54813
	Food	9.55043012	9.015711	0.54234	9.01571	9.605058	9.94260
Low Level	Resting	2.61445496	2.585235	0.05434712	2.58523	2.600160	2.64545
	Water	7.71146944	7.762471	7.80728	7.58578	7.762471	7.80728
	Food	7.844187	6.958250	0.60394	7.49894	7.717915	8.31764
High Level	Resting	2.625811	2.570396	0.13718	2.53368	2.585278	2.70707
	Water	5.900387	6.918310	1.03192	4.84172	6.089424	6.91831
	Food	1.803118	2.802206	1.04292	0.737055	2.247809	2.80220 6

Table XXXIV.

Electrophysiological Recordings (SQRT Transformed): Adult Female

Pairwise Comparisons [a]

Exposure	(I) Stimuli	Exposure	(J) Stimuli	Mean Difference (I-J)	Sig.[c]
Control	Resting	Control	Water	-1.0864	**0.0118***
			Food	-7.1437	**0.0003***
		Low Level	Resting	-0.2078	0.9002
			Water	-5.3048	0.0685
			Food	-5.4375	0.0655
		High Level	Resting	-0.2191	0.8788
			Water	-3.4937	0.1104
			Food	1.2684	0.6811
	Water	Control	Food	0.1156	**0.0004***
		Low Level	Resting	1.4649	0.6095
			Water	1.4648	0.1024
			Food	1.4643	0.0971
		High Level	Resting	1.2686	0.5648
			Water	1.2686	0.1982
			Food	1.6900	0.3143
	Food	Low Level	Resting	6.9360	**0.0418***
			Water	1.8390	0.3360
			Food	1.7062	0.3640
		High Level	Resting	6.9246	**0.0319***
			Water	3.6500	0.1025
			Food	7.7473	**0.0258***
Low Level	Resting	Low Level	Water	-5.0970	**0.0005***
			Food	-5.2297	**0.0004***
		High Level	Resting	-0.01136	0.9937
			Water	-3.2859	0.1223
			Food	0.8113	0.5879
	Water	Low Level	Food	-0.1327	0.3494
		High Level	Resting	5.0857	0.0569
			Water	1.8111	0.2895
			Food	5.9084	**0.0431***
	Food	High Level	Resting	5.2184	0.0543
			Water	1.9438	0.2650
			Food	6.0411	**0.0413***
High Level	Resting	High Level	Water	-3.2746	**0.0006***
			Food	0.8227	**0.0095***
	Water	High Level	Food	4.0973	**0.0004***

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: SQRTmV.

c. Adjustment for multiple comparisons: Bonferron

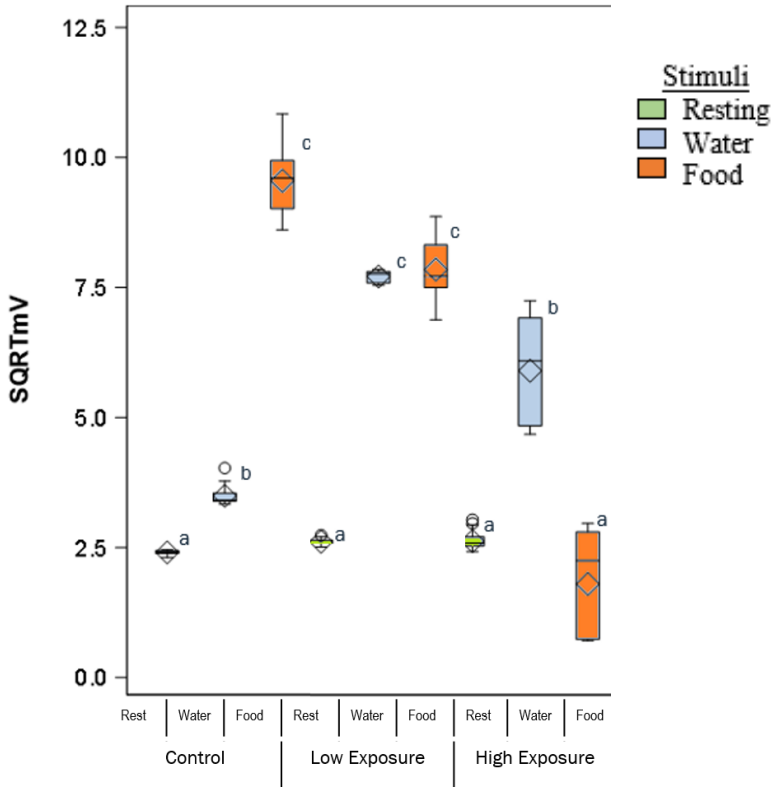

Fig. XI. Electrophysiological Recordings by Exposure Group: Adult Female. Neuron responsiveness of each exposure group of adult females to three conditions: resting (green), water stimuli (blue), and food slurry (orange).
[a], [b], [c] are significantly different at the .05 level.

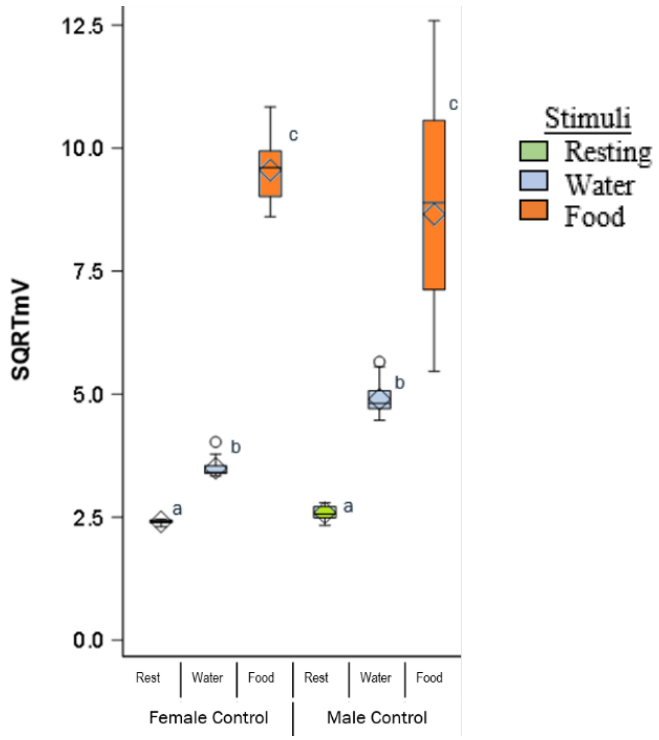

Fig. XII. Electrophysiological Recordings: Sex Comparison of Control. Neuron responsiveness of adult female and adult male controls to three conditions: resting (green), water stimuli (blue), and food slurry (orange).
[a], [b], [c] are significantly different at the .05 level.

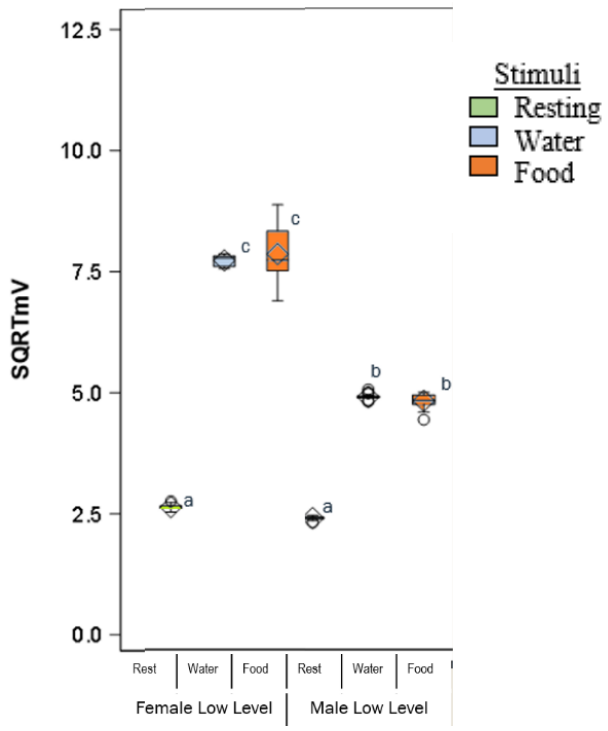

Fig. XIII. Electrophysiological Recordings: Sex Comparison of Low-Level. Neuron responsiveness of adult female and adult male low-level exposure groups to three conditions: resting (green), water stimuli (blue), and food slurry (orange). [a], [b], [c] are significantly different at the .05 level.

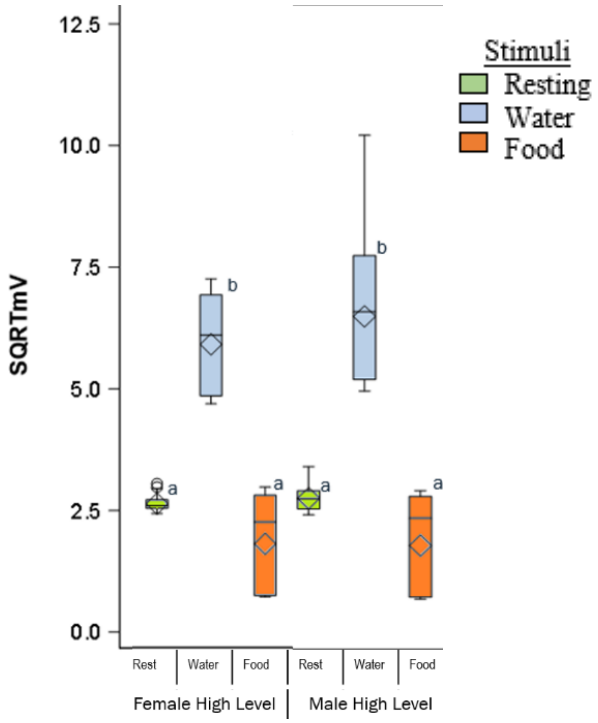

Fig. XIV. Electrophysiological Recordings: Sex Comparison of High-Level. Neuron responsiveness of adult female and adult male high-level exposure groups to three conditions: resting (green), water stimuli (blue), and food slurry (orange). [a] and [b] are significantly different at the .05 level.

Experiment III: Development

Table XXXV.

Survival Analysis: Adult Male

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.267	2	.875
Breslow (Generalized Wilcoxon)	.096	2	.953
Tarone-Ware	.018	2	.991

Test of equality of survival distributions for the different levels of Intervention.

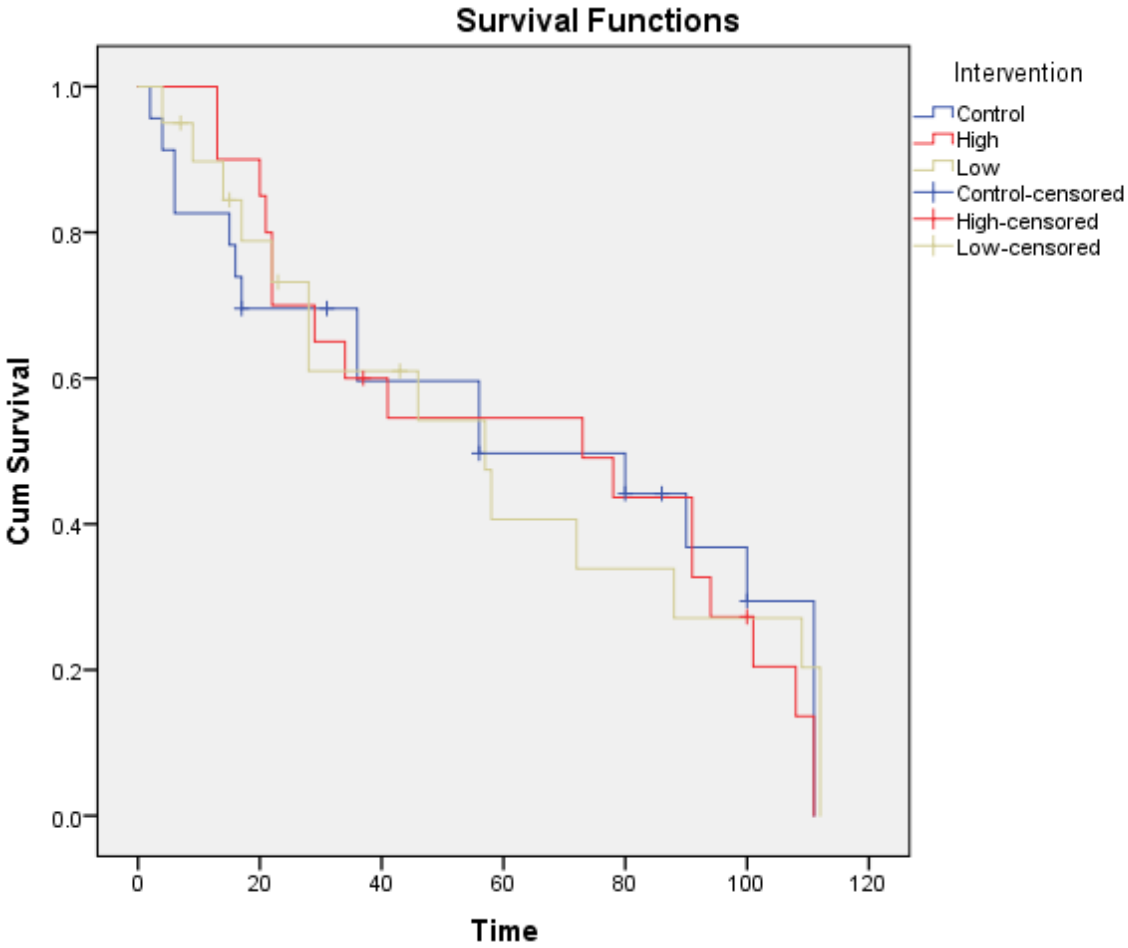

Fig. XV. Survival Analysis: Adult Male. Survival of adult males controls (blue), low-level exposure (yellow), and high-level exposure (red) over time in days. Cross hatch marks indicate a censored event, defined as a molt related death.

Table XXXVI.

Survival Analysis: Adult Female

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.676	2	.713
Breslow (Generalized Wilcoxon)	.114	2	.944
Tarone-Ware	.074	2	.964

Test of equality of survival distributions for the different levels of Intervention.

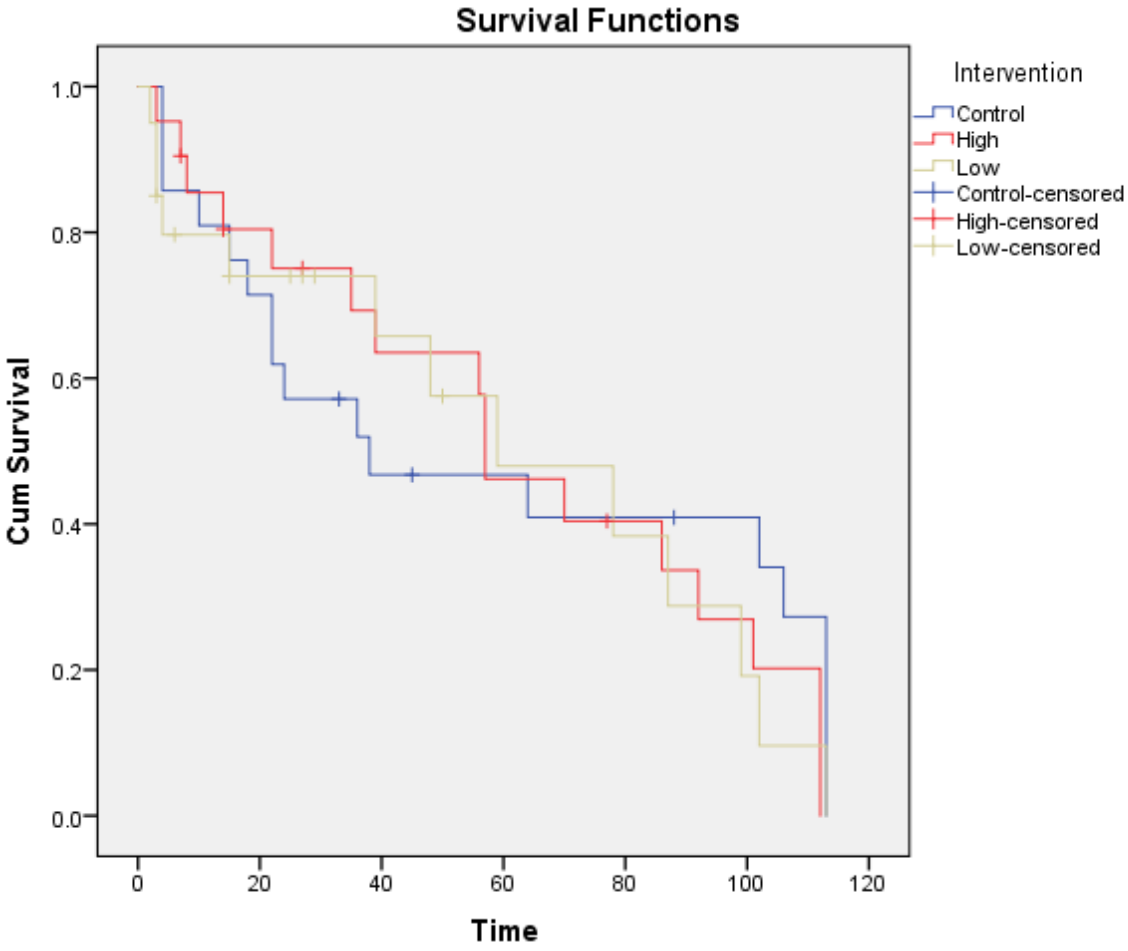

Fig. XVI. Survival Analysis: Adult Female. Survival of adult females controls (blue), low-level exposure (yellow), and high-level exposure (red) over time in days. Cross hatch marks indicate a censored event, defined as a molt related death.

Table XXXVII.

Survival Analysis: Juvenile Male

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	6.240	2	**.044***
Breslow (Generalized Wilcoxon)	3.220	2	.200
Tarone-Ware	4.390	2	.111

Test of equality of survival distributions for the different levels of Intervention.

Table XXXVIII.

Survival Analysis: Juvenile Male

Pairwise Comparisons

	Exposure	Control		High		Low	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	Control			1.892	.169	.088	.766
	High	1.892	.169			8.784	**.003***
	Low	.088	.766	8.784	**.003***		

*. The mean difference is significant at the .05 level.

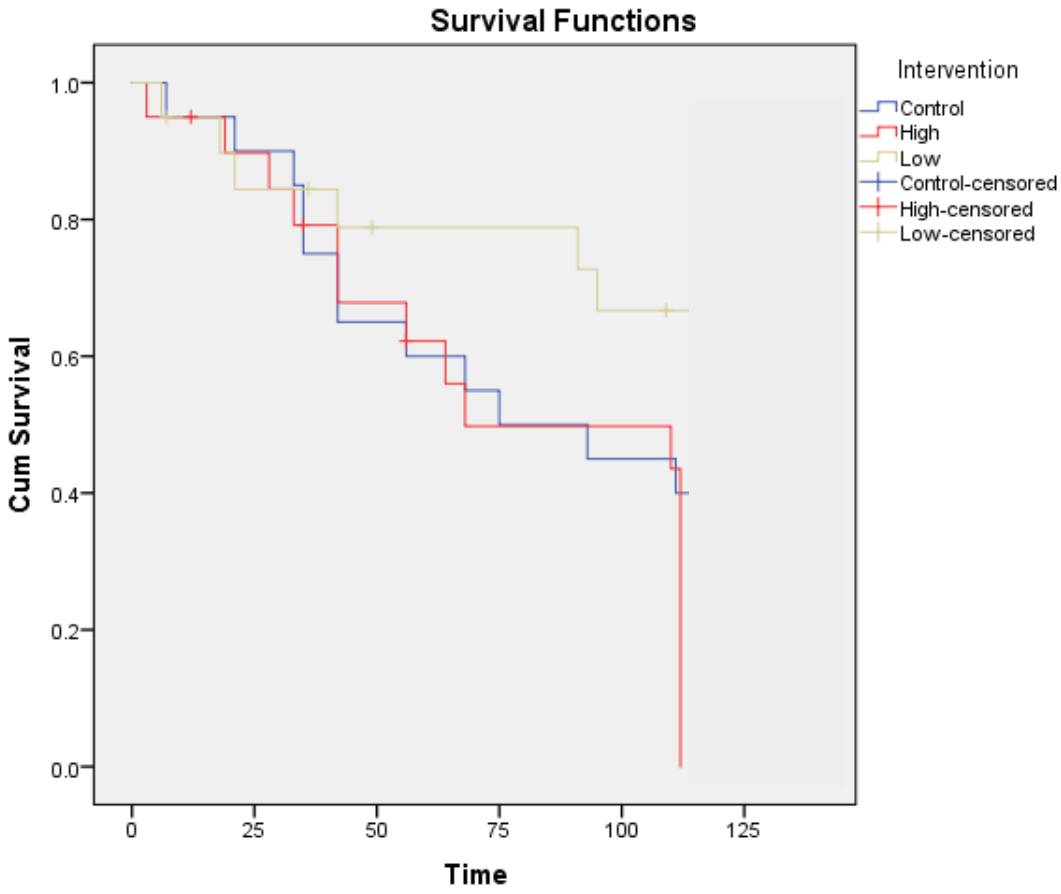

Fig. XVII. Survival Analysis: Juvenile Male. Survival of juvenile males controls (blue), low-level exposure (yellow), and high-level exposure (red) over time in days. Cross hatch marks indicate a censored event, defined as a molt related death. High-level exposure has significantly lower survival outcomes relative to the low-level exposure group at the .05 level

Table XXXIX.

Survival Analysis: Juvenile Female

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	20.551	2	**.000***
Breslow (Generalized Wilcoxon)	19.410	2	**.000***
Tarone-Ware	20.083	2	**.000***

Test of equality of survival distributions for the different levels of Intervention.

*. The mean difference is significant at the .05 level.

Table XL.

Survival Analysis: Juvenile Female

Pairwise Comparisons

		Control		High		Low	
	Exposure	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	Control			7.413	**.006***	6.635	**.010***
	High	7.413	**.006***			16.936	**.000***
	Low	6.635	**.010***	16.936	**.000***		
Breslow (Generalized Wilcoxon)	Control			6.567	**.010***	4.857	**.028***
	High	6.567	**.010***			15.855	**.000***
	Low	4.857	**.028***	15.855	**.000***		
Tarone-Ware	Control			7.040	**.008***	5.710	**.017***
	High	7.040	**.008***			16.524	**.000***
	Low	5.710	**.017***	16.524	**.000***		

*. The mean difference is significant at the .05 level.

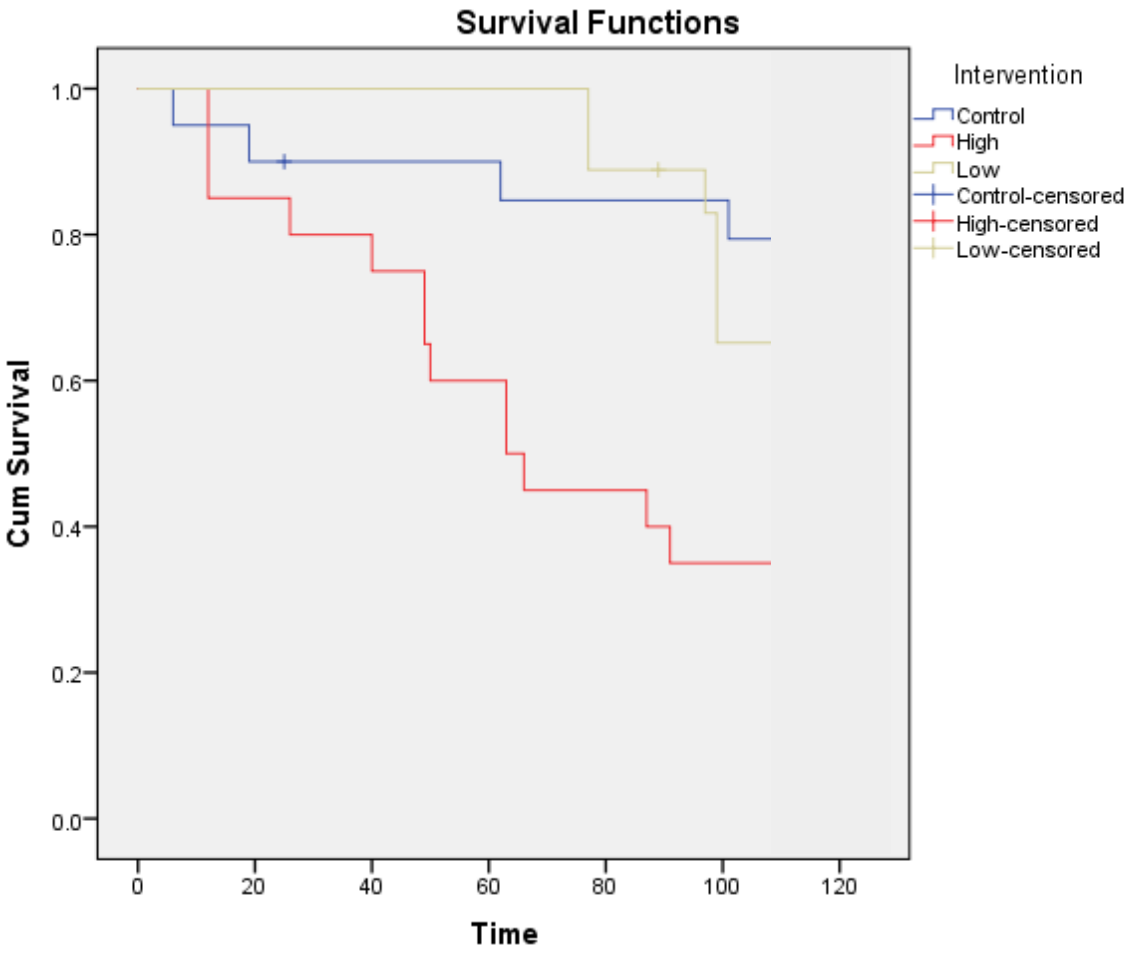

Fig. XVIII. Survival Analysis: Juvenile Female. Survival of juvenile females controls (blue), low-level exposure (yellow), and high-level exposure (red) over time in days. Cross hatch marks indicate a censored event, defined as a molt related death. High-level exposure has significantly lower survival outcomes relative to the control and low-level exposure group at the .05 level.

Table XLIII: Growth of Adult Females: Pairwise Comparisons[a]

Week	(I) Exposure	(J) Exposure	Mean Difference (I-J)	Sig.[b]	95% Confidence Interval for Difference[e]	
					Lower Bound	Upper Bound
.00	High	Low	-1.512	.057	-3.056	.033
		Control	-.270	1.000	-1.797	1.256
	Low	High	1.512	.057	-.033	3.056
		Control	1.241	.149	-.285	2.767
	Control	High	.270	1.000	-1.256	1.797
		Low	-1.241	.149	-2.767	.285
2.00	High	Low	-1.578*	**.045***	-3.130	-.026
		Control	.089	1.000	-1.444	1.622
	Low	High	1.578*	**.045***	.026	3.130
		Control	1.667*	**.029***	.132	3.202
	Control	High	-.089	1.000	-1.622	1.444
		Low	-1.667*	**.029***	-3.202	-.132
3.00	High	Low	-1.672*	**.032***	-3.234	-.110
		Control	-.237	1.000	-1.781	1.307
	Low	High	1.672*	**.032***	.110	3.234
		Control	1.435	.078	-.112	2.982
	Control	High	.237	1.000	-1.307	1.781
		Low	-1.435	.078	-2.982	.112
4.00	High	Low	-1.619*	**.042***	-3.197	-.041
		Control	-.264	1.000	-1.820	1.291
	Low	High	1.619*	**.042***	.041	3.197
		Control	1.355	.111	-.209	2.918
	Control	High	.264	1.000	-1.291	1.820
		Low	-1.355	.111	-2.918	.209
5.00	High	Low	-1.663*	**.038***	-3.258	-.069
		Control	-.303	1.000	-1.872	1.266
	Low	High	1.663*	**.038***	.069	3.258
		Control	1.360	.115	-.220	2.941
	Control	High	.303	1.000	-1.266	1.872
		Low	-1.360	.115	-2.941	.220
6.00	High	Low	-1.618*	**.050***	-3.235	-.002
		Control	-.267	1.000	-1.853	1.319
	Low	High	1.618*	**.050***	.002	3.235
		Control	1.352	.126	-.248	2.951
	Control	High	.267	1.000	-1.319	1.853
		Low	-1.352	.126	-2.951	.248
8.00	High	Low	-1.732*	**.039***	-3.399	-.066
		Control	-.389	1.000	-2.014	1.237
	Low	High	1.732*	**.039***	.066	3.399
		Control	1.344	.150	-.308	2.995
	Control	High	.389	1.000	-1.237	2.014
		Low	-1.344	.150	-2.995	.308
10.00	High	Low	-1.877*	**.030***	-3.614	-.140
		Control	-.342	1.000	-2.013	1.329
	Low	High	1.877*	**.030***	.140	3.614
		Control	1.535	.097	-.188	3.258
	Control	High	.342	1.000	-1.329	2.013
		Low	-1.535	.097	-3.258	.188

Based on estimated marginal means

*. The mean difference is significant at the .05 level. b. Adjustment for multiple comparisons: Bonferroni.

Table XLIV: Growth of Adult Females- Estimates[a]

Exposure	Week	Mean	Std. Error	df	95% Confidence Interval	
					Lower Bound	Upper Bound
High	.00	6.034	.444	61.379	5.146	6.921
	2.00	6.378	.446	62.328	5.487	7.268
	3.00	6.130	.448	63.908	5.234	7.026
	4.00	6.111	.452	65.826	5.209	7.014
	5.00	6.141	.456	68.139	5.230	7.051
	6.00	6.119	.462	71.646	5.197	7.041
	7.00	6.203	.468	74.960	5.270	7.137
	8.00	6.112	.474	78.070	5.168	7.056
	9.00	.[b]	.	.	.	.
	10.00	6.132	.488	85.444	5.163	7.102
	11.00	6.108	.496	89.898	5.123	7.093
	12.00	5.973	.504	94.010	4.973	6.972
	13.00	5.976	.511	97.774	4.962	6.990
	14.00	6.020	.532	111.879	4.965	7.075
	15.00	6.024	.562	132.097	4.913	7.136
	16.00	5.521	.606	164.385	4.325	6.718
Low	.00	7.545	.444	61.379	6.658	8.432
	2.00	7.955	.447	63.009	7.063	8.848
	3.00	7.802	.450	64.976	6.903	8.701
	4.00	7.730	.457	68.647	6.819	8.642
	5.00	7.804	.463	72.135	6.881	8.727
	6.00	7.738	.471	76.330	6.801	8.675
	7.00	7.722	.479	81.430	6.768	8.676
	8.00	7.844	.490	87.719	6.870	8.818
	9.00	7.847	.505	97.148	6.845	8.850
	10.00	8.010	.520	105.941	6.979	9.040
	11.00	7.387	.535	115.381	6.327	8.446
	12.00	7.517	.555	128.291	6.419	8.615
	13.00	7.450	.574	139.839	6.316	8.583
	14.00	7.207	.591	149.958	6.039	8.375
	15.00	7.352	.608	158.662	6.152	8.552
	16.00	7.586	.631	172.652	6.340	8.832
Control	.00	6.304	.433	61.379	5.438	7.170
	2.00	6.288	.436	62.900	5.417	7.159
	3.00	6.367	.440	65.171	5.489	7.245
	4.00	6.376	.444	67.320	5.490	7.261
	5.00	6.443	.448	69.880	5.549	7.338
	6.00	6.386	.453	72.290	5.484	7.288
	7.00	6.173	.458	75.190	5.261	7.085
	8.00	6.500	.466	79.642	5.574	7.427
	9.00	6.492	.474	84.446	5.550	7.434
	10.00	6.474	.480	87.703	5.521	7.428
	11.00	6.518	.491	94.109	5.544	7.492
	12.00	6.571	.504	102.181	5.572	7.570
	13.00	6.541	.520	112.637	5.511	7.571
	14.00	6.505	.550	134.339	5.417	7.593
	15.00	6.732	.578	153.769	5.590	7.875
	16.00	6.825	.621	184.739	5.600	8.049

b. This level combination of factors is not observed, thus the corresponding population marginal mean is not estimable.

Growth

Table XLI.

Growth of Adult Males

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure	2	56.685	.002	.998
Week	15	316.202	13.166	**.000***

a. Dependent Variable: Weight.

*. The mean difference is significant at the .05 level.

Table XLII.

Growth of Adult Females.

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure	2	65.816	3.246	**.045***
Week	15	316.152	2.624	**.001***
Exposure x Week	29	316.130	1.797	**.008***

a. Dependent Variable: Weight.

*. The mean difference is significant at the .05 level.

Table XLV.

Growth of Juvenile Males

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Exposure	2	53.178	1.275	.288
Week	14	468.581	12.237	.000

a. Dependent Variable: Weight.

Table XLVI.

Growth: Juvenile Females

Type III Tests of Fixed Effects[a]

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	57.647	222.530	.000
Exposure	2	54.047	.893	.415
Week	14	598.609	15.698	.000

a. Dependent Variable: Weight.

Gonad Mass

Table XLVII.

Gonad Mass: Adult Male

ANOVA

Gonad Mass

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	2	.000	.380	.687
Within Groups	.036	28	.001		
Total	.036	30			

Table XLVIII.

Gonad Mass: Adult Female

ANOVA

Gonad Mass

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.005	2	.003	.919	.417
Within Groups	.050	18	.003		
Total	.055	20			

Table XLIX.

Gonad Mass: Juvenile Male

ANOVA

Gonad Mass

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	2	.000	.329	.723
Within Groups	.003	22	.000		
Total	.003	24			

Table L.

Gonad Mass: Juvenile Female

ANOVA

Gonad Mass

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.005	2	.002	1.518	.237
Within Groups	.043	28	.002		
Total	.048	30			

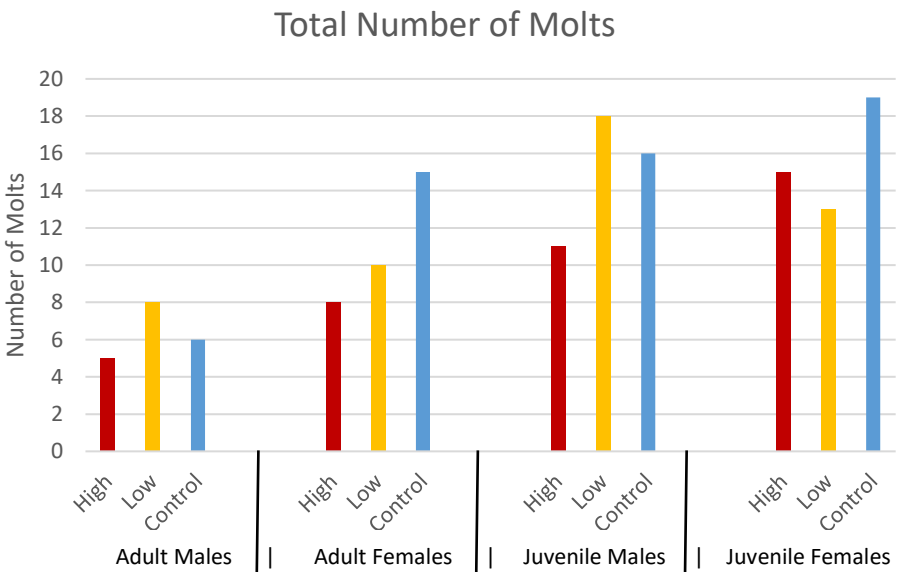

Fig. XIX. Total Number of Molts. Total number of molts of adult males, adult females, juvenile males, and juvenile females of the control (blue), low-level exposure (yellow), and high-level exposure groups (red).

Figure XX.

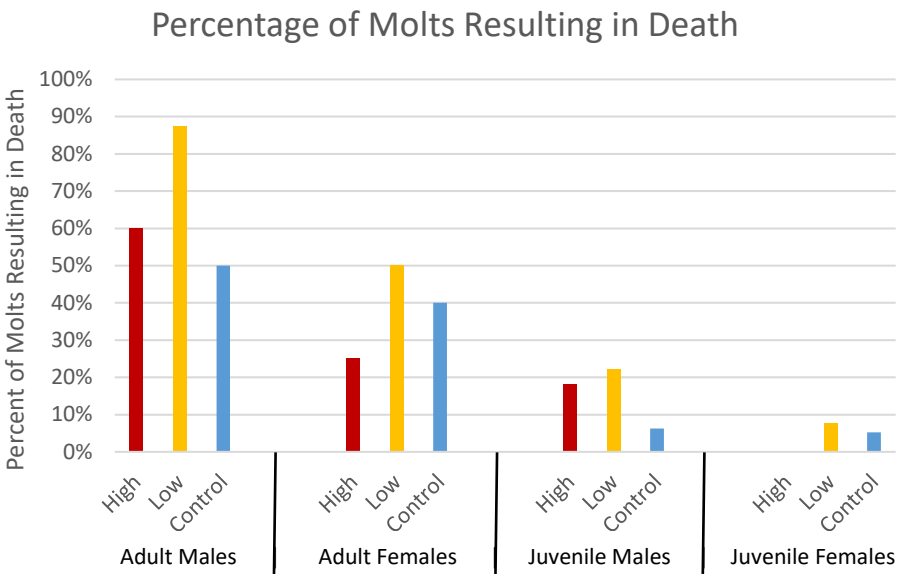

Fig. XX. Percentage of Molts Resulting in Death. Percentage of molting adult males, adult females, juvenile males, and juvenile females that did not survive the molting event. Depicted by group: control (blue), low-level exposure (yellow), and high-level exposure groups (red).

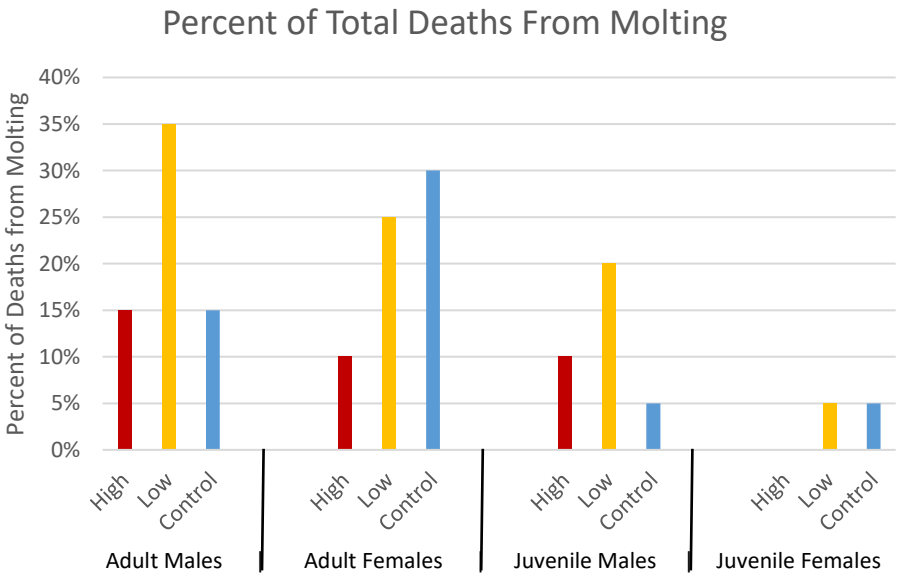

Fig. XXI. Percentage of Total Deaths from Molting. Percentage of adult males, adult females, juvenile males, and juvenile females that died from a molting related event. Depicted by group: control (blue), low-level exposure (yellow), and high-level exposure groups (red).

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