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Subthreshold Toxic Effects of Atrazine and Three Degradates on Behavior in Procambarus clarkii

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Subthreshold Toxic Effects of Atrazine and Three Degradates on Behavior in *Procambarus clarkii*

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Dedication

I would like to dedicate this thesis to Sara Starkey, my youngest sister and dearest friend. Thank you for always being my greatest supporter, for your undying friendship and love, as well as for the countless times you've made my day brighter simply with a smile or a laugh. Your quick wit, kindness, strength, and most of all your resilience inspire me on a daily basis. You've always been and always will be my person.

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Abstract

Subthreshold toxic effects of atrazine and three degradates on behavior in *Procambarus clarkii*

By Carol B. Starkey

Atrazine is among the most heavily applied pesticides worldwide, and recent evidence suggests that it may be unsafe at environmental levels. It is a known endocrine disruptor and a suggested neurotoxin. The US EPA Maximum Contaminant Level (MCL) is 3 µg/L for human ingestion and 200 µg/L for limited human exposure. Several environmentally persistent degradates have been identified, including deethylatrazine (DEA), deisopropylatrazine (DIA) and hydroxyatrazine (HA). No MCLs have been established for these degradates, although some are suggested toxins. Thus, there remains concern for the risk associated with the presence of atrazine and its degradates in the environment. Currently little data exist describing sublethal effects of atrazine and its degradates. Fortunately, toxicological research has evolved past dependency on mortality measures to incorporate sophisticated behavioral studies that can elucidate the effects of sublethal exposure to toxins. The goal of this research was to use such parameters to quantify the subthreshold (below the level at which harm is immediately detected) toxic effects of atrazine, DEA, DIA and HA on learning and behavior in *Procambarus clarkii* (red swamp crayfish), a sensitive bioindicator species. Crayfish were placed in an aquatic T-maze (classic method to test cognitive ability) with a food reward in a side arm. Various behavioral endpoints were recorded over repeated trials, including time to reward, time to reward arm, percent of crayfish to not locate reward arm, time spent in the under cover area, and time needed to complete ~180° turnarounds. Concentrations of the chemicals tested represented an environmentally realistic range. Each treatment level of atrazine, DEA, DIA and HA resulted in significant deficits compared to control for at least two behavioral endpoints. Furthermore, a nonmonotonic dose-response was observed for atrazine. Based on these results, crayfish exposed to atrazine and each degradate at low, biologically relevant doses had impaired boldness and explorative behavior compared to control. Thus, crayfish exposed to these contaminants in natural environments will have impaired abilities to locate food, find mates, and avoid predation, which will have a profound impact on their ability to survive. These implications extend beyond crayfish, a keystone species, to include any species that rely on them as a food source or as a source of vital energy to the ecosystem via their roles as detritus shredders.

Chapter 1. Introduction and Background

1.1. Introduction

Non-point source pesticide contamination from agricultural areas is one of the greatest causes of surface water pollution worldwide (Gangbazo *et al*., 1999). Atrazine is the most heavily used herbicide in the United States (Rebich *et al*., 2004; Weiner & Sandheinrich, 2010), with an average of 51 million pounds (23.13 million kg) of the active ingredient applied per year (Graziano *et al*., 2006). It is degraded in the environment through several biotic and abiotic mechanisms to form many degradation products, or degradates, predominantly deethylatrazine (DEA), deisopropylatrazine (DIA), and hydroxyatrazine (HA) (Battaglin *et al*., 2003; Lerch *et al*., 1998). Atrazine and its degradation products can persist for years and are among the most frequently detected contaminants in aquatic systems (Seeger *et al*., 2010; Rebich *et al*., 2004; Lerch *et al*., 1998; Battaglin *et al*., 2003).

The US Environmental Protection Agency has found that short-term human exposure to atrazine at concentrations greater than 3 µg/L, the USEPA's Maximum Contamination Level (MCL) for human consumption, may cause heart, kidney, and lung congestion, low blood pressure, weight loss, adrenal gland damage, and muscle spasms; long term exposure to concentrations above the drinking water MCL has been associated with degeneration of muscles and retinas, cardiovascular damage, and even cancer (Graziano *et al*., 2006). Water samples frequently exceed this MCL value (Battaglin *et al*., 2003; Wu *et al.,* 2010). The US also has an MCL set for limited human exposure, which is 200 µg/L (Hayes E. , 1993). It is not uncommon for water samples to occasionally exceed this concentration, particularly immediately after herbicide application in the spring and early summer (Kolpin *et al*., 1997; Battaglin *et al*., 2000). No health advisory values have been established in the US for atrazine's degradation products, although some are suggested toxins (Ralston-Hooper *et al*., 2009). However, the European

Union has a maximum permissible concentration of 0.1µg/L for atrazine and an allowed maximum of 0.5 µg/L for the combined total concentrations of atrazine and its degradation products (European Council, 1998). The EU banned the use of atrazine in 2004 due to its potential to contaminate groundwater at concentrations exceeding the allowed maximum, even when used appropriately (European Commission, 2003).

Due to the frequent use of atrazine and to the environmental persistence of it, as well as of its degradation products, there is reason to be concerned about the risks associated with the presence of atrazine, DEA, DIA and HA at environmental levels. Currently, there are little data on the sublethal effects of atrazine and its degradates at low, environmentally realistic concentrations. Fortunately, toxicological research has evolved past dependency on classic measures of mortality to incorporate sophisticated and more sensitive behavioral studies, which allow one to elucidate the effects of subthreshold (below the level at which harm may be immediately detectable) exposure to toxins.

The purpose of this research was to use such parameters to quantify the effects of various subthreshold doses of atrazine (including MCLs), DEA, DIA, and HA on behavior and learning in *Procambarus clarkii,* the Red Swamp Crayfish, a sensitive bioindicator (Alcorlo *et al*., 2006) and keystone species (Gutiérrez-Yurrita & Montes, 1999) that has recently been used as a model organism in ecotoxicological research. Crayfish were placed in an aquatic T-maze, a well established test of various forms of cognitive ability, consisting of three arms with a desirable reward (food) placed near one of the ends of a side arm. Several behavioral endpoints were recorded over repeated trials, including time to reward, time to reward arm, percent of crayfish to not locate reward arm, time spent in under cover area, and time needed to complete ~180° turnarounds. It was hypothesized that exposure to subthreshold doses of these toxins would result

in learning and behavioral deficits, such as impaired ability to locate food reward and reward arm over repeated trials, as well as lethargy, decreased exploration, and decreased motor skills. Impairments in learning and behavior may be an early indicator of chemical toxicity and may be used to predict chronic toxicity (Saglio & Trijasse, 1998). The results of this research may also be useful in determining how atrazine, DEA, DIA and HA are likely to affect other, more difficult to study species. Such deficits extend beyond the lab and will likely affect crayfishes' ability to survive, i.e. locate food and potential mates and avoid predation in natural environments. Furthermore, impairments in learning and behavior of crayfish are likely to have detrimental effects on other species that depend on crayfish either as a food source or as a vital source of energy (via their roles as detritus shredders) to the ecosystem. Thus, the ecological impact of atrazine and its degradates' presence in the environment extend well beyond the effects observed in crayfish.

1.2. Statement of the Problem

Data are currently lacking on the subthreshold toxic effects of atrazine. Furthermore, comparative studies on the subthreshold toxicity of atrazine's primary degradation products DEA, DIA, and HA have yet to be performed. These chemicals are detected frequently and are relatively persistent in the environment (Seeger *et al*., 2010; Rebich *et al*., 2004; Lerch *et al*., 1998; Battaglin *et al*., 2003), and it has been found that degradation products are often as toxic, if not more so, than the parent compound (Sinclair & Boxall, 2002). Thus, there is great need to be concerned about the risks associated with sublethal, environmentally relevant concentrations of atrazine, DEA, DIA and HA.

1.3. Atrazine and its Risks

Atrazine, or 2-Chloro-4-ethylamino-6-isopropylamino-s-triazine, is a photosynthesis inhibitor that is used to control certain annual broadleaf weeds. It is mostly used to treat corn, especially in the Midwestern United States, but it is also commonly used in sorghum and sugarcane (Rohr & McCoy, 2010). It is a member of the class of herbicides known as triazines, which contain a symmetrical ring consisting of three nitrogens and three carbons in an alternating sequence; other examples of such herbicides include propazine and simazine. Atrazine is further classified as an s-triazine, or 1,3,5-triazine (Figure 1.1), meaning the nitrogens in the triazine ring are located at the 1,3, and 5 positions (USEPA, 1996).

Atrazine is among the most heavily applied pesticides worldwide, and according to Wiener and Sandheinrich (2010) it is the most heavily used herbicide in the United States. An average of 51 million pounds, or 23.13 million kg, of the active ingredient is applied per year (Graziano *et al*., 2006). Such high rates of use have led to the widespread contamination of surface and ground waters, as well as to atmospheric dispersal. Atrazine has one of the highest frequencies of detection among pesticides in freshwater sources (USEPA, 1990; Gilliom *et al*., 2006; Benotti *et al*., 2009), and has been detected in arctic ice, seawater, ambient air and fog at locations far from agricultural and city areas (Chernyak *et al*., 1996; Jablonowski & Schaffer, 2011). There has been some debate recently as to the dangers of inputing such large amounts of atrazine into the environment. Recent evidence suggests that concentrations frequently detected may pose risks to humans and other organisms, as well as a threat to the environment (Wu *et al*., 2010).

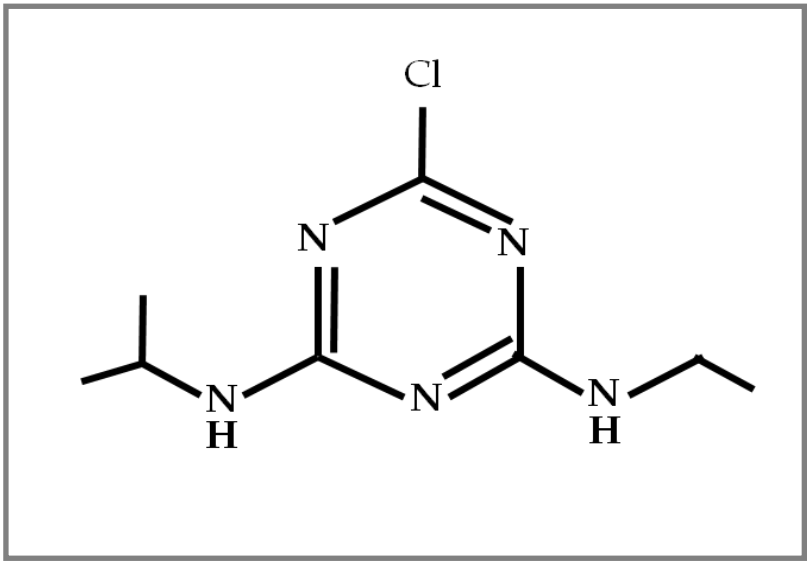

Figure 1.1. Chemical structure of atrazine, 2-Chloro-4-ethylamino-6-isopropylamino-s-triazine. It is a member of the class of herbicides known as triazines, which contain a symmetrical ring consisting of three nitrogens and three carbons in an alternating sequence, and is further classified as an s-triazine, meaning the nitrogens in the triazine ring are located at the 1,3, and 5 positions (USEPA, 1996).

Classic measures for testing toxicity, such as LC 50 (concentration at which 50% lethality is observed in test organims) yield relativley high values for the lethality of atrazine. For example, the LC50 after 96 hours of exposure to atrazine was determined to be 1,600 µg/L, 1,500 µg/L, and greater than 3,000 µg/L for the unicellular algae *Psudokirchneriella subcapitata,* and the amphipods *Hyalella azteca*, and *Diporeia spp*, respectively (Ralston-Hooper *et al*., 2009). These values drop considerably after chronic exposure, for example: 240 µg/L for *Diporeia spp* after 21 days of exposure (Ralston-Hooper *et al*., 2009). Elevated concentrations such as these are normally only detected in surface waters immediately following herbicide application or in tailwater pits, which collect runoff from fields. Concentrations as high as 224 µg/L have been detected in US Midwestern streams, while a much higher level of 2300 µg/L has been measured in tailwater pits in midwestern agricultural areas (Kolpin *et al*., 1997; Battaglin *et al*., 2000). However, as mentioned previously, these elevated values are normally detected within the first few weeks following herbicide application in late spring to summer. Concentrations detected for the remainder of the year, fall to spring, tend to be much lower, with annual detected averages $\leq$ 5 µg/L (Wu *et al*., 2010). Thus, measures of LC50 do not accurately represent toxic effects that may actually result from much lower, environmentally relevant concentrations. Therefore, there is a need to determine the toxicity of such chemicals at concentrations commonly detected in water samples throughout the year.

There is little evidence that atrazine causes direct mortality of water-dwelling animals at environmentally realistic concentrations; however, this is not evidence that atrazine does not have a sublethal toxic effect on exposed organisms. Fortunately, there has been a shift in toxicological research where sensitive behavioral and learning endpoints have been employed to determine subthreshold effects of various toxins on test organisms. Potential sublethal effects of

pesticides include alteration of the nervous system, biochemical changes, effects on reproduction, as well as potential chronic effects, which include a decrease in biomass and dry weight of adult animals (Rakotondravelo *et al*., 2006; Cook & Moore, 2008). A few examples of behavioral and learning endpoints used to test the sublethal effects of toxins include the following: swimming behavior of zebrafish (Steinberg *et al*., 1995) and goldfish (Saglio & Trijasse, 1998) to determine the subthreshold effects of atrazine, agonistic behavior of the crayfish *Orconectes rusticus* to test the sublethal effects of the herbicide metolachlor (Cook & Moore, 2008), and honey bee (*Apis mellifera* L.) performance of the classic proboscis extension reflex, which is part of a bee's feeding behavior, as well as honey bee performance in a T-tube maze test to assess sublethal effects of GM products and pesticides (Han *et al.*, 2010).

According to Lin *et al.* (2013) exposure to atrazine at low doses not associated with immediate signs of toxicity results in several behavioral abnormalities. Behaviorally, chronic exposure to atrazine creates deficits in motor coordination, impairs spontaneous locomotor activity, and also alters the spatial memory of rats (Bardullas *et al*., 2011). Several studies that combine behavioral and neurochemical analyses have found that exposure to atrazine results in behavioral deficits that most consistently coinside with alterations in dopaminergic systems, particularly the nigrostiatal system, regardless of protocal (Rodriguez *et al*., 2013; Lin *et al.*, 2013). In a study by Rodriguez *et al*. (2013) repeated atrazine exposure altered monoamine and monoamine metabolite levels in the striatus of Sprague-Dawley rats. Animals were sacrificed six days after their sixth and final aministration of 100 mg ATR/kg. It was found that striatal levels of dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) were decreased by 20 to 27% (Rodriguez *et al.*, 2013).

Interestingly, in a somewhat similar study, the directionality of alterations in dopamine, DOPAC, HVA and 5-HIAA levels were opposite or not significant. Short term oral exposure of C57BL/6 mice to atrazine at 125 and 250 mg/kg resulted in a 7.4% and 12.2% (respectively) increase in striatal levels of dopamine (Lin *et al*., 2013). 125 or 250 mg/kg atrazine exposure also elevated HVA levels; however, there was no significant change in DOPAC levels from control. Treatment with atrazine did not alter serotonin levels, while there was an increase in striatal 5-HIAA levels for both treatment levels of atrazine (Lin *et al*., 2013). Furthermore, Lin *et al*. (2013) observed that levels of DOPAC, HVA, 5-HIAA, and norepinephrine in the prefrontal cortex were all elevated by atrazine exposure, as well. Although results of alterations in monoamine systems may vary, which may be due to differences between species or more simply a result of variable dose regimens, such results agree in supporting the role of atrazine as a neurotoxin that directly affects the dopaminergic system. It has also been suggested that changes in striatal levels of 5-HIAA without alterations in serotonin levels may be due to possible effects of atrazine on the regulation of catabolic enzymes, such as aldehyde dehydrogenase and (or) monoamino oxidase (Rodriguez *et al*., 2013). Many animal models, particularly mammalian models, of atrazine neurotoxicity test concentrations that are oftentimes greater than levels detected in the environment. However, changes in locomotor activity are also observed when using low atrazine levels (1 ug/kg/day) that are environmentally realistic (Belloni *et al*, 2011). Thus, changes in locomotor activity and other behaviors may be the most sensitive indication of atrazine toxicity (Rodriguez *et al*., 2013).

Concentrations ranging .005 - .08 uM, or 1.08 - 17.25 µg/L, have been shown to be clastogenic, or to cause chromosomal damage, to chinese hamster (*Cricetulus griseus*) ovary cells after 48 hours of exposure. Damage to chromosomes was assessed by measuring the

coeffeicient of variation (the ratio of the standard deviation to the mean) and the percent of chromosomes present in larger chromosome distribution peaks. Furthermore, atrazine concentrations comparable to public water supply levels revealed its potential to induce chromosome breaks (Biradar & Raybur, 1995).

There is also strong evidence to support that atrazine is an endocrine disruptor at concentration frequently detected in the environment. In a study by Hayes *et al*. (2002), exposure to concentrations less than or equal to 0.1 µg/L induced hermaphroditism and demasculinized male African Clawed frogs' (*Xenopus laevis*) larynges. Furthermore, males had a 10X decrease in plasma testosterone levels as a result of exposure to 25 µg/L of atrazine. At doses ranging from 0.1-200 µg/L gonadal abnormalities were observed. For example, 16-20% of frogs had multiple gonads, with the highest number of gonads in an individual animal being six. Hayes *et al.* (2002) stated that such abnormalities had never been seen before in control animals in over 10, 000 observations over a period of six years. In a later study it was documented that among male frogs born and reared in water contaminated with 2.5 µg/L atrazine, 10% matured to have female sex characteristics, reduced testosterone levels, lowered sperm levels, as well as eggs present in their testes. These animals exhibited female mating behavior and attracted normal males, with which they would procreate (Hayes *et al*., 2010). Furthermore, it has been demonstrated that atrazine demasculinizes gonads of males in many vertabrate species, resulting in lesions of the testes, which are linked with lowered germ cell numbers (in amphibians, teleost fish, reptiles, and mammals), and it abets limited or total feminization in fish, amphibians, and reptiles. These observed effects were statistically significant, specific, and occurred across classes of vertebrates (Hayes *et al*., 2011).

Meta-analysis is the systematic analysis of previous research in order to examine similar end points to gain broad conclusions, add strength for hypotheses, and/or develop an estimation of general effects (USEPA, 2009). Rohr and McCoy (2010) performed a qualitative meta-analysis on 125 independently published research studies on the effects of atrazine. They concluded that atrazine can both retard and accelarate amphibian metamorphosis and that atrazine decreased size near or directly at metamorphosis in over 88% of studies. In 70% of studies atrazine altered, at mininum, a single aspect of gonadal morphology, it consistently altered function of gonads, and it altered spermatogenesis (2/2 studies) as well as levels of sex homones in nearly 86% of studies included in their meta-analysis (Rohr & McCoy, 2010). Amphibians need to meet a mininum size before metamorphose can occurr, and once they meet this size they may accelerate their deveopment and metamorphosis if in a stressful environment or delay metamorphose if in a good environment (Wilbur & Collins, 1973). Metamorphosis is primarily controlled by hormones of the thyroid and corticosterone (Larson *et al*., 1998). Therefore, it is reasonable to assume that endocrine disruption can result in inappropriate timing of metamorphosis. This research provides further evidence for the endocrine disrupting potential of atrazine.

Atrazine elevated locomotor activity levels of amphibians and fish (> 92% of studies). In 80% of the analyzed fish studies, but none of the amphibian studies, a nonmonotonic dose response was observed. Atrazine at low concentrations stimulated hyperactivity in fish, while higher concentrations caused reduced activity. However, in amphibians, hyperactivity was normally observed at all concentrations tested, while higher concentrations would likely result in lowered levels of activity (Rohr & McCoy, 2010). It is worth noting that hyperactivity was observed hundreds of days following the end of atrazine exposure, and there was evidence that

animals failed to recover from exposure, which suggests nonreversible effects (Rohr & Palmer, 2005; Rohr & McCoy, 2010). These effects of atrazine on amphibian and fish activity are in agreemant with changes observed in locomotor activity in mammals. Atrazine caused mammal hyperactivity by competing with inhibitory neurotransmitter gammaaminobutyric acid receptors, via changing monoamine turnover and via neurotoxicity of the dopaminergic system (Rodriguez *et al*., 2007; Das *et al*., 2001; Rohr & McCoy, 2010). Furthermore, it lowered antipredator behaviors in six out of seven studies (Rohr & McCoy, 2010). Decreased antipredator behaviors coupled with elevated hyperactivity may lead to elevated encounter rates with possible predators (Skelly, 1994), resulting in decreased survival rates.

Atrazine has also been documented to lower fish olfactory skills in five out of five studies reviewed in a dose-dependent manner (Moore & Waring, 1998; Moore & Lower, 2001; Tierney *et al*., 2007). Rohr and McCoy (2010) stated that exposure to atrazine by itself was associated with 21 out of 27 end points of lowered immune function, and it in combination with other pesticide(s) was linked to 12 out of 16 of such end points. Furthermore, similar to atrazine's effects on fish and amphibian immunity, exposure to atrazine was consistently linked to an increase in end points for infection in fish and amphibian at environmentally realistic concentrations. The herbicide elevated trematode, nematode, viral and bacterial infections. An elevation in 13 (of 16 total) end points for infection was observed (Rohr & McCoy, 2010).

Needless to say, although atrazine is unlikely to cause the direct death or immediate detectable harm in exposed organisms there is great need to be concerned about the health risks to humans and other organisms at sublethal levels, in addition to the threat that it poses to the environment at large. Sublethal harm, such as impaired immune function, neurotoxicity, or endocrine disruption, takes a large toll on the overall health and reproductive success of animals

affected, which may result in a domino-like effect that alters the overall health of the ecosystem which said animals inhabit.

1.4. Atrazine's Presence in Aquatic Systems

Roughly 75% of all pre-emergent herbicides used in the US are administered to row crops within the corn belt area, an area consisting of 12 states within the Midwestern US (Gianessi & Puffer, 1985). The Midwestern region provides 60% and 75% of the nation's soybean and corn crops, respectively. Once atrazine is applied to crops in late spring and early summer a large portion of it disperses into nearby streams, rivers, and other bodies of water via runoff (Periera & Hostettler, 1993).

According to the US Geological Survey (USGS) huge concentrations of pesticides are flushed from the soil and are then transported through surface water as pulses, which result from rainfall events following spring/summer application (Thurman *et al*., 1991; Periera & Hostettler, 1993). The Ohio, Missouri and Des Moines Rivers are the largers contributors of atrazine to the Mississippi River (Pereira & Hostettler, 1993). Vital factors affecting the distribution and ultimate fate of pesticides in the Mississippi River Basin include pesticide biogeochemical properties; the geographic location of the crop and the length of application time; sorptic (adsorption and absorption) capacity and type of soil; tillage practices; and differences in conditions, such as climate, season, and hydrology. Generally, compounds that have relatively lengthy half-lives (see Table 1.1 for atrazine's half-life values) and are relatively soluble in water are most easily transported in runoff from agricultural areas or in groundwater (Pereira & Hostettler, 1993). Because atrazine is relatively water soluble, ~33 mg/L, (Table 1.1), it has great

potential for leaching into ground water, transport in surface waters, aerial transport, as well as occurrence in precipitation (Thurman *et al*., 1991).

Under the Safe Drinking Water Act, water systems may technically be in compliance with the annual mean limit of 3 µg/L for atrazine when calculations are based on a running annual average. The EPA only requires that samples be taken by water systems one to four times per year to ascertain whether or not they comply with the MCL standard (Wu *et al.,* 2010). Thus, high spikes in concentrations in the spring and/or summer are easily minimized by low detections during other parts of the year and elevated concentrations that last for a few weeks can be easily overlooked based on timing of sampling. However, increased monitoring has shown that some systems in fact exceeded the MCL standard (Wu *et al., 2010*).

The National Resources Defense Council's (NRDC) original 2009 report determined that surface waters of the Midwestern and Southern US are pervasively contaminated by atrazine. Every watershed sampled (40 total) had detectable levels of atrazine, while 25 of these had mean concentrations greater than 1 µg/L, which is the level at which primary production by nonvascular, aquatic plants is lowered (Wu *et al.,* 2009). It was also stated that watersheds in Nebraska, Missouri and Indiana had the 10 highest peak concentrations and that some of these had, at minimum, a single sample of incredibly high concentrations, ranging from 50-200 µg/L (Wu *et al*., 2009). Additionally, drinking water data have revealed high levels of contamination by atrazine in drinking water in public water systems. Of samples taken in 2003 and 2004 in 139 water systems, greater than 90% had measurable levels of atrazine, and 54 of these water systems had a one-time peak concentration greater than the drinking water MCL value (Wu *et al*., 2009).

In the NRDC's 2010 update data from the Ecological Watershed Monitoring Program collected by Syngenta, the manufacturer of atrazine, from 2007 to 2008 were analyzed (Wu *et al*., 2010). Samples were taken from 20 watersheds located in the states of Ohio, Nebraska, Indiana, and Illinois and were collected in early spring through the summer or fall (USEPA, 2009). The NRDC also analyzed the Atrazine Monitoring Program drinking water data, which were collected from 2005 through 2008. These samples were collected from 153 public water systems located in Ohio, Kentucky, Louisiana, Missouri, North Carolina, Indiana, Iowa, Illinois, Florida, Texas, and California (USEPA, 2013). The updated analyses showed continued and extensive contamination in treated, or water deamed ready for human injestion, and untreated water. Eighteen of the watersheds analyzed had atrazine concentration spikes greater than 20 µg/L, while nine reached concentrations at 50 µg/L or greater (Table 1.2). The highest recorded maximum concentration was 147.65 µg/L, which occurred in May of 2008 in the Big Blue River watershed (Nebraska), and this 'peak' lasted a total of twelve days, with a range of concentrations from 27.92-147.65 µg/L. Such lengthy spikes are not uncommon (Wu *et al*., 2010). Sixteen of the twenty watersheds sampled (Table 1.2) had an annual average concentration greater than 1 µg/L, which, as stated previously, is the level at which a reduction in primary production in non-vascular, aquatic plants in observed. This is likely to have detrimental effects on the stream ecosystem itself, as well as other ecosystems near it (USEPA, 2006).

Furthermore, concentrations of atrazine in samples of treated water have been documented to exceed 3 µg/L. 67 of the water systems analyzed by the NRDC had concentrations greater than this value (Wu *et al.,* 2010). For example, one of Ohio's water systems (Piqua City Public Water System) had a maximum concentration of 59.57 µg/L in treated water, while its maximum untreated concentration was 84.80 µg/L. Six total systems had

concentrations of atrazine exceeding the EPA's standard for drinking water, and were located in Missouri (1), Ohio (2), Indiana (1), Illinois (1), and Kansas (1) (Wu *et al.*, 2010). Therefore, it is not uncommon for concentrations greater than the EPA's MCL for drinking water to be detected in public water supplies that have been treated and deemed safe for human consumption. This further emphasizes the need to develop a better understanding of of the risks associated with exposure to atrazine at concentrations frequently detected in the environment as well as in treated water.

Table 1.1. Physical and chemical characteristics of atrazine. Note the variation in atrazine's aqueous half-life due to varying water conditions.		
Characteristic	**Value**	**Comments**
Molecular Weight	215.7 g/mol[A]	
Water solubility	33 mg/L[B]	At 22 °C
Koc	25.3 – 155.0 g/ml[C]	Range of values represent various soil types
pKa	1.7[D]	
Aqueous half-life	52.5 days[A]	Lake water
	43 days[A]	River water
	56.3 days[A]	Marine water
	26.2[A]	Ground water
	34.5 days[A]	Distilled water
Soil half-life	15 – 100 days [E]	Varies with soil properties.

[A] Konstantinou *et al*., 2001; [B] Periera & Hostettler, 1993; [C] Ciba-Geigy Coorperation, 1994; [D] Xu *et al*., 1999; [E] Protzman *et al*, 1999

Table 1.2: Maximum and Annual Average Atrazine Concentrations in 20 Midwestern US Watersheds, 2007 – 2008 (Data from Wu *et al*., 2010). Eighteen of the watersheds analyzed had atrazine concentration spikes greater than 20 µg/L, while nine reached concentrations at 50 µg/L or greater. The highest recorded max concentration was 147.65 µg/L (Big Blue River watershed, 2008); this 'peak' lasted 12 days (not shown). Such lengthy spikes are not uncommon (Wu *et al*., 2010).

Watershed	Date	Sample #	Max (µg/L) (Date)	Annual Avg. (µg/L)
Spring Creek, IL	2007	124	3.25 (6/2/2007)	0.36
Iroquois River, IL	2007	139	12.69 (4/26/2007)	0.84
Horse Creek, IL	2007	105	42.77 (5/16/2007)	2.41
Vermilion River, North Fork, IN	2007	101	12.15 (4/25/2007)	0.43
Little Pigeon Creek, IN	2007	88	2.95 (8/4/2007)	0.33
	2008	174	27.12 (5/3/2008)	1.10
Little Pigeon Creek, subwatershed, IN	2007	61	1.44 (4/27/2007)	0.30
	2008	155	15.10 (5/3/2008)	1.11
South Fabius River, MO	2007	102	91.60 (6/2/2007)	5.02
	2008	47	62.75 (6/3/2008)	2.03
South Fabius River, MO upstream	2008	192	78.20 (6/3/2008)	1.98
Youngs Creek, MO	2007	120	16.18 (4/26/2007)	2.33
	2008	225	56.60 (5/26/2008)	2.73
Seebers Branch, South Fabius River, MO	2007	124	65.73 (4/26/2007)	2.05
	2008	220	144.69 (5/12/2008)	4.20
Main South Fabius River, MO	2007	121	42.97 (5/4/2007)	2.00
	2008	219	33.60 (6/3/2008)	1.43
Long Branch, MO	2007	126	21.08 (4/26/2007)	3.18
	2008	225	37.83 (6/9/2008)	2.02
Long Branch, MO, main	2008	207	36.23 (5/25/2008)	2.80
Big Blue River, Upper Gage, NE	2008	173	147.65 (5/8/2008)	9.12
Big Blue River, Upper Gage, NE; adjacent site	2008	184	116.03 (5/7/2008)	8.45
Muddy Creek, NE	2008	175	67.81 (5/30/2008)	2.49
Big Blue River, Lower Gage, NE	2008	200	82.80 (5/22/2008)	2.07
Big Blue River, Lower Gage, NE; adjacent site	2008	188	32.90 (5/24/2008)	2.32
Lower Muddy Creek, NE	2008	153	50.00 (5/30/2008)	2.25
Licking River, North Fork, OH	2007	128	9.90 (5/16/2007)	0.62

1.5. Atrazine Degradation

Once released into the environment, atrazine is degraded through several biotic and abiotic mechanisms. Atrazine's fate in aqueous systems is very much determined by the s-triazine ring, which makes it resistant to microbial attack (Howard, 1991). Therefore, chemical degradation may be more important than biodegradation in the environment. Three of the most frequently encountered primary degradation products of atrazine include deethylatrazine (DEA), or 2-amino-4-chloro-6-(isopropylamino)-s-triazine; deisopropylatrazine (DIA), or 2-amino-4-chloro-6-(ethylamino)-s-triazine; and hydroxyatrazine (HA), or 2-hydroxy-4-(ethylamino-6-(isopropylamino)-s-triazine, (Figure 1.2) (Prosen & Zupančič-Kralj, 2005). DEA and DIA are formed by microbial and chemical degradation via the N-dealkylation at the fourth carbon (Giardina *et al*., 1982; Behki & Khan, 1986). Loss of the ethyl group is preferred to loss of the isopropyl group in aerobic soils, and deethylation has been shown to be two to three times faster than deisopropylation (Mills & Thurman, 1994). Atrazine is also degraded to HA as a result of hydrolysis at the second carbon.

Atrazine has been found to be stable for 30 days in laboratory conditions at 25°C and in a pH range of 5 to 9 (Ciba-Geigy Corporation, 1994). However, once in the environment, atrazine's half-life varies wildly depending on environmental conditions (see table 1.1). Organic molecules in surface water and soil are adsorbed or bound to humic substances, such as fulvic acid, humin, and humic acids. S-triazines, incuding atrazine, may bind via electron transfer to humic substances, proton tranfer, or hydrogen bonding (Wang *et al*., 1991; Senesi *et al*., 1995; Sposito *et al*., 1996; Martin-Neto *et al*., 2001). The presence of humic substances and binding to them may act to catalyze abiotic transformation of triazines to their 2-hydroxy degradates (Stenvenson, 1982). For example, at 25°C and pH 4 atrazine's half-life was calculated to be 244

days, but with the addition of 2% humic acid this value dropped to 1.73 days (Li & Feldbeck, 1972). The rate of hydrolysis is also oftentimes increased by extremes in pH, sorption to soil colloids, as well as the presence of other photosensitizing compounds (Lerch *et al*., 1998). According to Lerch and Li (2001), hydroxylated degradation products, particularly HA, constitue nearly 90% of bound residues of triazines in soil.

Triazine photodegradation in the upper layer of soil and surface waters results from either direct (substance absorbs UV energy) or indirect, humic substance-sensitized, photolysis. Photolysis of atrazine is restricted to wavelengths no greater than 300 nm in water (Pape & Zabik, 1970). Konstantinou *et al*. (2001) found that photodegradation in distilled water was a result of direct photolysis. The photoreaction site of atrazine involves the chlorine in the second position, which supports dechlorination and hydroxyderivative formation as the major pathway in direct photolysis (Barcelo *et al*, 1993; Torrents *et al*., 1997; Konstantinou *et al*., 2001). However, N-dealkylated products, including DEA and DIA, were detected in natural water samples as well, which suggested that indirect photolysis was due to the presence of dissolved organic matter. Photodegradation was decreased in natural waters, compared to distilled and ground water. It was concluded that this may be due to quenching of sunlight by organic matter or scattering of sunlight by microorganisms or sediment particles suspended in the water column. Photodegratdation rates in all natural waters tested followed a first order degradation curve, or exponential decay (Konstantinou *et al*., 2010).

In the same study by Konstantinou *et al.* (2010), it was determined that photodegradation rates were faster in soil than in water samples; thus, the sensitizing effect, during which other media constituents absorb light energy and then transfer energy to the chemical, must be greater than the scattering effect in soils. Photolysis occurs only within a shallow surface zone of soils;

how deep this zone is depends on soil characteristics. The vertical depth of direct photolysis on surface soil is restricted to a region of roughly 0.2 – 0.3 mm, while indirect photolysis may occur at depths greater than 0.7 mm for outdoor experiments (Hebert & Miller, 1990). Humic acids may act as sensitizers in creating reactive intermediates. These reactive species may diffuse as deeply as 1 mm, depending on soil characteristics such as moisture, depth, and porosity (Konstantinou *et al*., 2010). Metal oxides in the soil absorb sunlight radiation and may elevate degradation by reaction with reactive intermediates such as singlet oxygen, hydroxyl radicals and hydrogen peroxide, a mechanism known as semiconductor photochemistry. Additionally, the chemical properties of sorbed compound are quite different from their properties in solution, which makes it difficult to predict what effects may actually result from sorption in various conditions (Konstantinou *et al*., 2010).

The use of atrazine worldwide has potentially contributed to the global distribution of already known microorganisms that have newly-evolved catabolic enzymes or new microorganisms with atrazine catabolizing abilities. Due to the full oxidation of carbons in the ring of atrazine there are limitations to its usefulness as an energy source (Radosevich *et al*., 1995). However, due to the presence of both nitrogens and carbons, atrazine's catabolic susceptibility is increased under nitrogen and carbon limited conditions (Ralebitso *et al*., 2002).

Degradation of s-triazine herbicides by bacteria involves hydrolytic reactions, which are catalyzed by amidohydrolases, a type of hydrolase that acts upon amide bonds. Strains of bacteria belonging to the following genera: *Pseudomonas, Arthrobacter, Chelatobacter, Agrobacterium, Rhodococcus, Stenotrophomonas, Pseudaminobacter* and *Nocardiodes* have thus far been characterized (Topp *et al*., 2000; Rousseaux *et al*., 2001; Hernandez *et al*., 2008a, 2008b, 2008c). *Pseudomonas sp.* strain ADP (Atrazine Degrading Pseudomonas), the most

studied atrazine degrading strain, was initially isolated from a spill site in Minnesota and employs atrazine as its sole source of nitrogen, mineralizing it in the process (Mandelbaum *et al*., 1995). Thus, *Pseudomonas sp.* strain ADP is the model organism for studying s-triazine degradation (Seeger *et al*., 2010). As a result, the degradation pathway of atrazine by this bacterium has been fully described (de Souza *et al*., 1998). This process consists of four steps, including: dehalogenation, N-dealkylation, deamination, and cleavage of the ring. The upper s-triazine catabolic pathway feeds into the cyanuric acid cycle (Figure 1.3). The genes that encode the enzymes for this process have been encoded and are known as *atzA, atzB,* and *atzC* genes (de Souza *et al*., 1998). The lower s-triazine catabolic pathway then mineralizes cyanuric acid to eventually form carbon dioxide and NH_3 (Figure 1.4) (Martinez *et al*., 2001). The genes that encode the enzymes for the lower catabolic pathway are known as *atzD, atzE, and atzF* (Strong *et al*., 2002). Additionally, it has been suggested that two of the atrazine degrading strains, *Pseudomonas sp*. strain ADP and *Agrobacterium radiobacter* J14a, are chemotactically attracted to atrazine (Park *et al*., 2003), and a study by Liu and Parales (2009) went even further to suggest that *Pseudomanoas sp*. strain ADP is chemotactically attracted to atrazine's metabolites, N-isopropylammelide and cyanuric acid, as well.

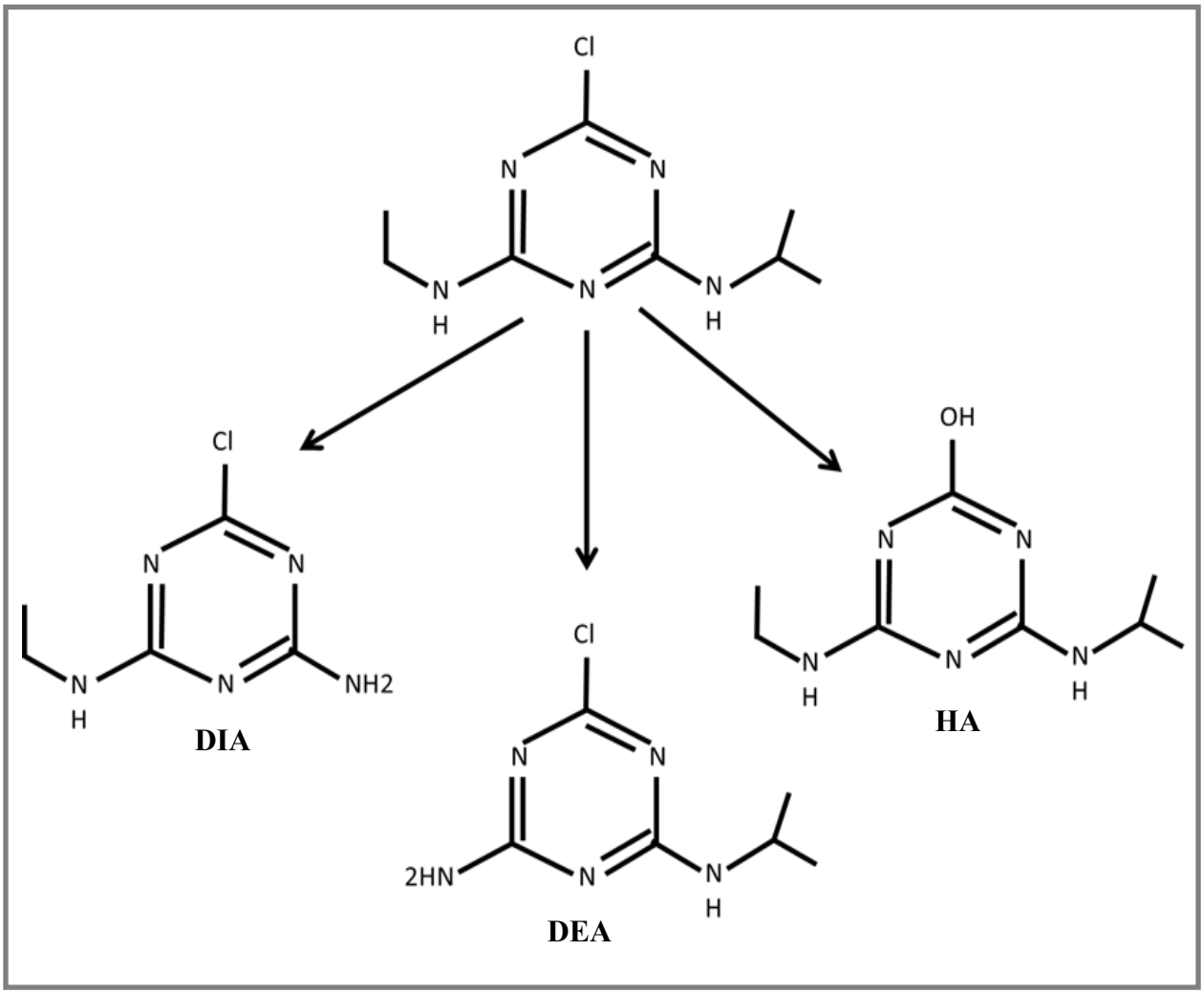

Figure 1.2. Atrazine's primary environmental degradation products are formed via various biotic and abiotic mechanisms. The N-dealkylated atrazine degradates include deisopropylatrazine, or DIA (left), and deethylatrazine, or DEA (bottom center), and the primary hydroxylated degradation product is hydroxyatrazine, or HA (right).

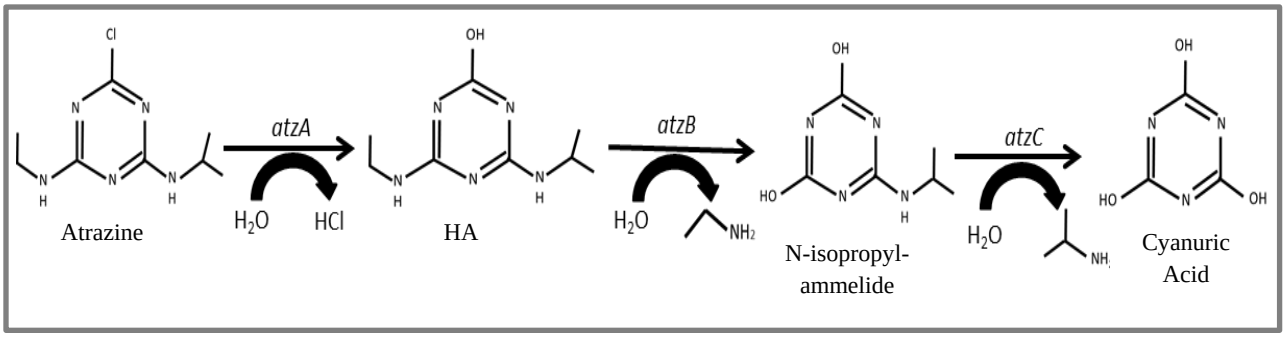

Figure 1.3. The upper atrazine catabolic pathway elucidated with *Pseudomonas sp*. strain ADP. This pathway degrades atrazine into cyanuric acid. The *atz* genes that encode enzymes for each reaction are indicated at each step.

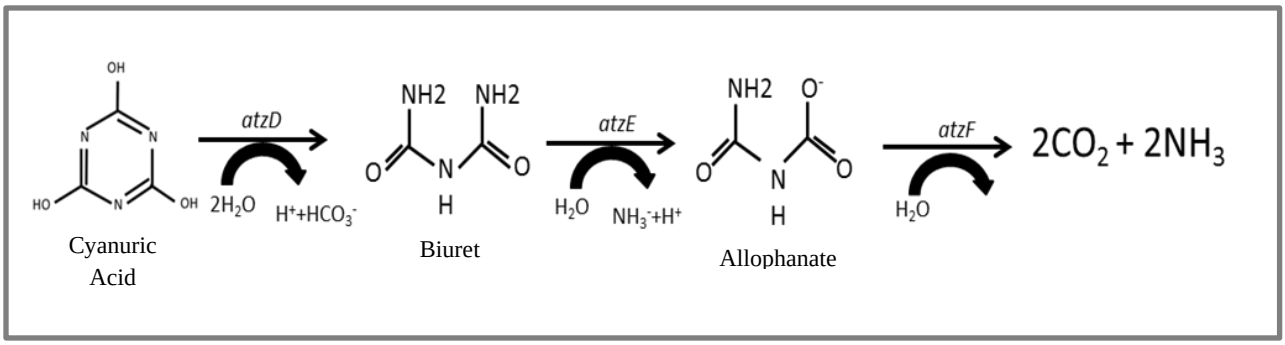

Figure 1.4. The lower catabolic pathway mineralizes cyanuric acid into carbon dioxide and NH_3. The *atz* genes that encode enzymes for each reaction are indicated at each step.

1.6. Deethylatrazine, Deisopropylatrazine, and Hydroxyatrazine

Atrazine is relatively persistent with a half-life ranging from 15 to 100 days in soil, depending on environmental conditions. Regardless, its degradation products constitute a significant portion of the total atrazine load (atrazine plus its stable degradates) found in rivers, streams, and ground water (Battaglin *et al*., 2003). Table 1.3 lists the percentage of samples taken by Battaglin *et al.* (2003) in Midwestern surface waters in which detections of atrazine, DEA, DIA and HA were at or above the reporting limit (0.05 µg/L), as well as their respective median and maximum concentrations taken during pre-emergence (after 50% or more of the corn crop was planted – May or June) runoff and post-emergence (after 90% or more of the soybean crop had emerged – June or July) runoff events. In a different study, DEA's maximum concentration was reported to be 7.5 µg/L, while DIA's and HA's were 7.4 µg/L and 3.7 µg/L, respectively in nearby sampling areas (Midwest US) (Lerch *et al*., 1998). Thus, it is not uncommon to find reports of degradation products' concentrations exceeding the MCL value of the parent compound.

Detection of atrazine was determined to be greater in pre-emergence than post-emergence samples, and the difference in concentrations between the two sampling events was statistically significant ($p < 0.05$) and positive (Battaglin *et al*., 2003). This trend is not surprising as atrazine is a pre-emergent herbicide; thus, its concentration is expected to be the highest immediately after its application and it is expected to then degrade via various routes. The opposite is true for atrazine's degradates' detection frequencies, which were larger in post-emergence samples. This is also makes coherent since as detection of degradation products is expected to increase as atrazine begins to be degraded in the environment.

Table 1.3. Summary of Atrazine, DEA, DIA, and HA in Pre-(after 50% or more of the corn crop was planted – May or June) and Post- Emergence (after application of post-emergence herbicides and after 90% or more of the soybean crop had emerged – June or July) Run-Off Samples (Data from Battaglin *et al*., 2003). Atrazine's detection and concentrations were significantly greater during Pre-Emergence, or immediately following application. DEA, DIA and HA detection frequencies increased during Post-Emergence, i.e. after the most recently applied atrazine had begun degrade in the environment.

	Pre-Emergence			**Post-Emergence**		
Chemical	**% Detection**	**Med. (µg/L)**	**Max. (µg/L)**	**% Detection**	**Med. (µg/L)**	**Max. (µg/L)**
Atrazine	100	4.07	172.2	98.7	2.69	34.8
DEA	92.0	0.41	2.67	94.7	0.54	3.66
DIA	86.7	0.32	2.34	92.1	0.39	2.17
HA	48.0	<0.05	12.4	54.0	0.27	4.43

According to Lerch *et al.* (1998), the proportion of DEA, DIA, HA, and atrazine to the total atrazine load in pre-plant (March and April) samples was 11.4%, 7.6%, 38.6% and 42.4% respectively, while DEA, DIA, HA, and atrazine constituted 13.6%, 8.5%, 14.4% and 61.1% respectively of the total atrazine load in post-plant (May to July) samples taken from northern Missouri streams. DEA and DIA's contribution to the total atrazine load did not change significantly between pre-plant and post-plant; however, HA's contribution was greatly reduced from 39% to 14% of the total atrazine load between pre-plant and post-plant. The largest change in atrazine load between these two sampling periods was an increase in the proportion of atrazine and a simultaneous decrease in the proportion of HA. Additionally, the median atrazine loads were elevated roughly seven fold from pre- to post-plant, which demonstrates the impact that annual atrazine use has on the total atrazine load in streams and rivers (Lerch *et al*., 1998). Levels of HA were similar to atrazine and were usually greater than DEA and DIA from late summer until the following spring. Levels of the parent compound increased dramatically during the initial six weeks following its application, which is fairly typical (Lerch *et al*., 1998). Because atrazine's degradates contribute such a large amount, over half in pre-plant samples, to the total atrazine load there is need to be concerned about the physical and chemical characteristics of DEA, DIA, and HA, as well as the potential for risks that may be associated with them.

HA is expected to be the least mobile in water and soil systems, compared to atrazine and its chlorinated degradation products, based on its physiochemical properties (Table 1.4) (Ciba-Geigy Corporation, 1994). K_{oc}, or the soil organic carbon-water partitioning coefficient, is the ratio of the mass of a chemical that is adsorbed in the soil per unit mass of organic carbon in the soil per the equilibrium chemical concentration in solution (Vryas *et al*., 2007). It is the

distribution coefficient (K_d) normalized to total organic carbon content. K_{oc} values are useful in predicting the mobility of organic soil contaminants; higher K_{oc} values correspond to less mobile organic chemicals. A very high K_{oc} implies that the chemical is strongly adsorbed onto soil and organic matter and thus does not move, or leach, through the soil efficiently. Thus, HA, which has a relatively high K_{oc} of 374 – 13,797, is not expected to leach into soil nearly as well as atrazine, DEA, or DIA and is transported in aqueous systems with greater proportions bound to suspended particulate matter. DEA, in contrast, is expected to be most mobile in the aquatic environment and has the greatest leaching potential based on its lesser K_{oc} and K_d values. K_{oc} and K_d values for atrazine and DIA are higher than those for DEA and are substantially less than those for HA (Table 1.4). Under field conditions, a mere 0.4% of applied [^{14}C]HA leached past the uppermost 24 cm of soil, while 13.0%, 16.6%, and 11.1% of [^{14}C]atrazine, [^{14}C]DEA, and [^{14}C] DIA, respectively leached beyond this point (Schiavon, 1988).

It has also been suggested that the adsorption of s-triazines to soil organic matter and clays is related to their pK_a values (acid dissociation constant). Atrazine, DEA, DIA, and HA are mildly basic compounds with pK_as of 1.7, 1.4, 1.5, and 5.2, respectively (Table 1.4) (Vryzas *et al*., 2007). S-triazines with a pK_a between 4 and 5 have stronger sorption to soil colloids than do those with a pK_a close to 2 (Lerch *et al*., 1998). Based on the low leaching potential and relatively high sorption of HA to soil, its potential to contaminate groundwater is low. DEA, DIA, and atrazine have a much greater potential to contaminate groundwater, therefore, for the opposite reasons. HA also has greater potential to contaminate surface water and surface soils than do atrazine and its chlorinated degradation products due to its chemical characteristics and the annual application of atrazine (Lerch *et al*., 1998); this is also partially due to the high half-life of HA, which was determined to be 121 days in western Tennessee soil (Table 1.4)

(Winkelmann & Klaine, 1991). In the same study the soil half-life of DEA was determined to be 26 days, while DIA's half-life was 17 days (Winkelmann & Klaine, 1991). It is important to keep in mind that half-lives vary greatly depending on the study and the environmental conditions under which it was performed. Thus far, aqueous half-lives for atrazine primary degradation products have not been found in the literature.

Unfortunately, the risk associated with atrazine's presence in the environment does not merely subside as it is degraded. Herbicide degradation products can be comparably as toxic as their parent compounds (Kolpin *et al*., 1998), and as explained previously, atrazine's degradation products are detected quite frequently and often at concentrations exceeding the US MCL for the parent compound. Currently, very little data exist on the toxicity of atrazine's degradation products, particularly at the sublethal level and especially for HA. However, recent studies have suggested that these degradation chemicals are toxic in their own rights. According to Laws *et al*. (2003), the EPA recently decided that DEA and DIA share a common mechanism of toxicity to atrazine as a result of their ability to suppress the luteinizing hormone (LH) ovulatory surge and have consequential effects on reproductive development and function in laboratory rats. Estrous cycle disruption in adult Long Evans and Sprague-Dawley rats has been reported as a result of 21 days of exposure to atrazine (75-300 mg/kg, oral gavage), which was very likely mediated through changes in neurotransmitter and hormonal control of the gonadal function (Cooper *et al*., 1996). Furthermore, hypothalamus concentrations of dopamine increased, while norpinephrine levels decreased as a result of atrazine exposure (Cooper *et al*., 1998). After single or multiple (three and 21 days) doses of atrazine, the estrogen-induced surge of LH and prolactin in rats with removed ovaries was diminished. Intravenous injection of gonadotropin-releasing hormone (GnRH) reinstated secretion of LH in rats, which provided further evidence for a central nervous

system-pituitary mode of action (Cooper *et al.*, 2000). Additionally, exposure to a mixture of atrazine and its metabolites, including HA, diaminochlorotriaze, DEA, and DIA, at concentrations as low as 0.09 mg/kg of body weight during late pregnancy resulted in persistent alterations in the development of mammary glands of female offspring of Long-Evans rats (Enoch *et al*., 2007). In a test of acute and chronic toxicity of atrazine, DEA and DIA on amphipods (*Hyalella azteca* and *Diporeia* spp) and a unicellular algae (*Pseudokirchneriella subcapitata*), the order of toxicity was determined to be atrazine > DEA > DIA (Ralston-Hooper *et al*., 2009). These results were in agreement with toxicity tests on algal species, which demonstrated that atrazine was more toxic than the chlorinated metabolites (Tchounwou *et al*., 2000; Kross *et al*., 1992). It is worth noting that HA was not included in these studies (Ralston-Hooper *et al*., 2009; Tchounwou *et al*., 2000; Kross *et al*., 1992). The EPA was prompted by the lack of effects data on atrazine's degradates to state that the toxicities of these chemicals are equivalent to that of the parent compound and continued to state that degradates should be taken into consideration for risk assessment purposes (USEPA, 2003). Therefore, it is necessary to determine the subthreshold toxic effects of not only atrazine, but also of its degradation products at environmentally realistic concentrations in order to build on our understanding of the consequences that may arise from the substantial annual use of this herbicide. Simply stating that levels of the parent compound decrease a few weeks after early summer application is insufficient and gives one the false impression that the risks associated with atrazine's use decreases as it is flushed away or degraded in the environment.

Table 1.4. Physiochemical characteristics of atrazine's primary environmental degradation products - DEA, DIA, and HA. K_{oc} - soil organic carbon-water partitioning coefficient, K_d – distribution coefficient, and pK_a – acid dissociation constant. Based on physiochemical properties, DEA and DIA are expected to be quite mobile in water systems and leach into ground water, with DEA being the most mobile. HA is expected to be the least mobile in water and soil systems; thus, it has low potential to contaminate ground water, but it has greater potential to contaminate surface water and soils than the N-dealkylated degradates.

Chemical	K_{oc} [A]	K_d [A]	pK_a [B]	**Soil half-life** [C]	**Water solubility** [B] **(22 °C)**
DEA	12.2 – 44.9	0.06 – 1.02	1.4	26 days	2700 mg/L
DIA	30 – 97	0.27 – 2.73	1.5	17 days	980 mg/L
HA	374 – 13,797	1.98 – 389	5.2	121 days	16 mg/L

[A] Ciba-Geigy Coorperation, 1994; [B] Vryzas *et al*., 2007; [C] Winkelmann & Klaine, 1991

** Please note that atrazine's physiochemical properties are listed in Table 1.1.

1.7. Hypotheses

It was hypothesized that exposure to environmentally realistic, subthreshold doses of atrazine, DEA, DIA, and HA would result in learning and behavioral deficits, such as lethargy and decreased motor skills in *Procambarus clarkii*.

Crayfish treated with various levels of atrazine, DEA, DIA and HA were expected to have impaired abilities to locate the food reward and reward arm over repeated trials, compared to untreated crayfish. Also, it was hypothesized that treatment groups would have a greater percentage of animals to not locate reward arm. Treatment groups were anticipated to spend more time in the under cover area, a covered section of the maze (directly behind where animals were initially placed into maze) that provided an area in which animals could more easily avoid detection by 'potential predators'. Such results would indicate impaired learning (assuming the effect of trial /day was significant for the control group for these variables), increased lethargy, and decreased boldness and explorative behavior as a result of exposure to subthreshold levels of atrazine, DEA, DIA and HA. Furthermore, it was hypothesized that treated animals would require more time to complete ~180° turnarounds, indicating impaired motor response due to exposure to toxicants. Additionally, it was predicted that the subthreshold order of toxicity for the parent compound and the chlorinated degradation products would be atrazine > DEA > DIA, based on previous tests of acute and chronic toxicity on amphipods and unicellular algae (Ralston-Hooper *et al*., 2009). Due to lack of information in the literature, no prediction was made as to how comparably toxic HA was to the other chemicals.

Chapter 2. Materials and Methods

2.1. Crayfish

Mixed sex adult *Procambarus clarkii*, Red Swamp Crayfish, ranging from approximately five to nine centimeters in length were supplied by Atchafalya Biological (Raceland, LA). The sex, rostrum to telson length (cm), initial (trial day one) weight, and final (trial 15) weight (g) of each animal was recorded. Animals were kept in a 12 hour light/dark cycle and were isolated into 2 L aquaria, each with a small shelter and a bed of pebbles. Crayfish were fed three small pellets of Ocean Nutrition brand Formula One Pellets every four to five days during the trial period, so as to prevent starvation as well as satiation.

2.2. Chemicals

Atrazine (98.9% purity), DEA (98.2% purity), DIA (99.3% purity), and HA (98.3% purity) were each obtained from ChemService, located in West Chester Pennsylvania. Stock solutions were made for each of the treatment chemicals by dissolving 15 mg of atrazine, DEA, DIA, or HA into 1 L of artificial fresh water. Artificial fresh water consisted of 1 tablespoon of Aquarium Pharmaceutical's Aquarium Salt per 1 gallon of carbon-filtered water. Stock solutions were refreshed every thirty days.

2.3. Treatments and Treatment Period

Ten to twelve crayfish were used for each treatment group, while the control group consisted of 17 animals. There was variation in the number of crayfish for many groups due to unexpected deaths or experimenter error. Each treatment animal was exposed to its respective treatment for 14 days (the treatment period) prior to the onset of trials. Controls were placed into

untreated aquaria for 14 days prior to trials, as well. The concentrations of solutions tested tested were as follows: for atrazine: 200 µg/L, 10 µg/L, 3 µg/L, and 0.5 µg/L; DEA: 10 µg/L and 0.5 µg/L; DIA: 10 µg/L and 0.5 µg/L; and HA: 10 µg/L and 0.5 µg/L. The concentrations tested for each chemical represent levels that are environmentally realistic. Specifically, testing atrazine at 200 µg/L (MCL for limited human exposure) and 3 µg/L (MCL for human consumption) was done to determine if these concentrations, currently considered safe by the EPA, have a subthreshold effect on learning and behavior. The remaining concentrations, 0.5 µg/L and 10 µg/L, were tested for each chemical in order to directly compare the effects of each degradate to the parent compound, as well to do pairwise comparisons. Treatments were made by using the appropriate stock solution to make a dilution (using artificial fresh water) to the concentration being tested and were stored in separate 2 L aquaria, in which a single crayfish was then placed.

2.4. Aquatic T-Maze Apparatus

Two T-mazes, modeled after McMahon *et al*. (2005) were made from 10 cm diameter PVC pipe. The entrance arm, which was 120 cm, was joined with the two side arms (55 cm) with a T-joint. Each arm was capped at the ends to make the apparatus watertight. The cap at the base of the entrance arm provided the under cover area. A cut, 3 cm in width, was made along the top of each arm so that the experimenter could view the crayfish. Other 'landmarks' of the maze included the start line, the met junction line, and the left and right exit junction lines. The food reward, indicated by the stars, was randomly assigned per crayfish to either the right or left side arm, and was placed approximately 25 cm past the exit junction line for that arm (Figure 2.5). The food reward consisted of a small piece of bologna sewn into a small mesh bag, which had a length x width of approximately1 $cm^{2.}$ The reward was placed in a baggy to prevent animals from consuming it, while also allowing them to receive sensory stimulation from the reward.

Each maze was cleaned every two to three days by removing the water via syphoning. The inside walls and caps at the end of the mazes were wiped down thoroughly and were rinsed with deionized water. Then, the mazes were wiped down again. The mazes were cleaned with deionized water, as opposed to using any chemicals, so as to prevent any unnecessary and undesired exposure to additional contaminants other than those being directly tested. Afterwards, the mazes were refilled with artificial fresh water until they were roughly half full, and the mazes were re-scented.

To scent the mazes one-third of a piece of bologna was placed into the entrance arm, left side arm, and the right side arm. Scenting the mazes served the purpose of diffusing the food reward scent throughout the maze so that when an animal was performing a trial it wasn't olfactorily detecting and locating the reward. The purpose of this test was that each crayfish would discover the food reward whilst exploring the maze, then potentially learn the location of the food and return to it during a later trial, presumably at faster rates over repeated trials if the animal learned the food reward's location.

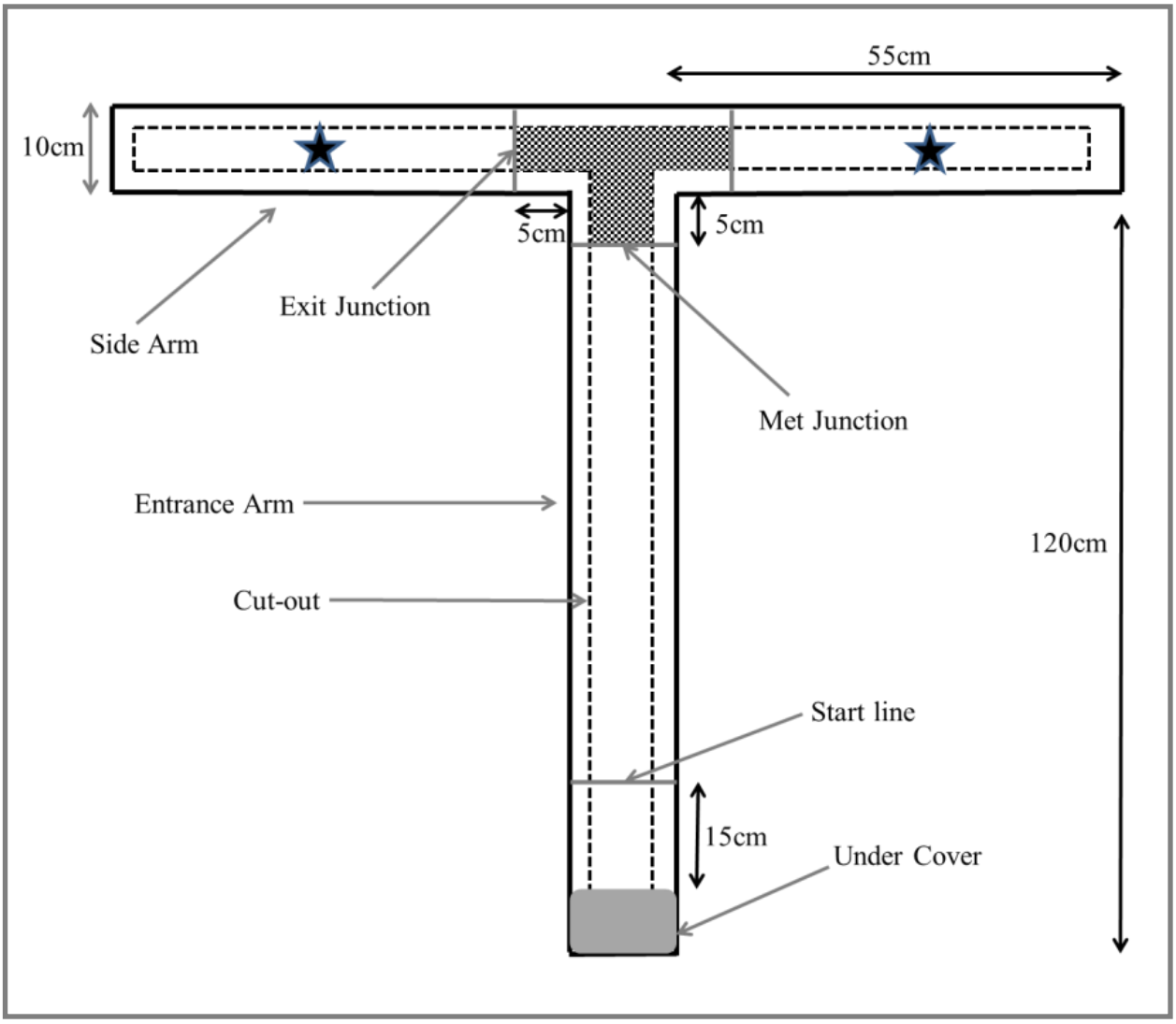

Figure 2.1. The T-maze consisted of an entrance arm, 120 cm, joined to two side arms (55 cm) with a T-joint and was capped at each end to make the apparatus water tight. Crayfish were placed in the start area and were viewed through the cut out as they navigated the maze. Animals could choose to navigate the maze by walking past the start line, toward the junction, then into a side arm where they may find the food reward, which was randomly assigned to either the left or right side arm. Crayfish could also remain, or return to, the start area and move to the under cover area where animals could avoid 'potential predators' and not explore.

2.5. Acclimation Period

The last five days of the treatment period also constituted the acclimation period. Thus, treatment days 10 – 14 corresponded to acclimation days 1 – 5. During an acclimation day, each animal was given thirty minutes to explore the maze, under similar conditions as a trial, but without a food reward and scenting of the maze. The purpose of allowing animals to explore the maze prior to trials was to familiarize them with the environment, thus reducing any potential stress that may have resulted from introducing crayfish to a novel environment on trial day one. Additionally, it has been suggested that crayfish prefer to explore identified environments (McMahon *et al*., 2005); therefore, allowing crayfish to habituate to the maze prior to trials served the purpose of decreasing inhibition of boldness to explore during early trials. Implementation of acclimation or habituation days is common for behavioral testing (Lin et al., 2013; Alvarex & Fuiman, 2005; Cook & Moore, 2008).

2.6. Trial Period

Trials were recorded on Sony HVR-A1U or Canon Vixia HFM52 high-definition cameras, viewed in iMovie ^{TM}, compressed, and then stored on DVDs. The trial period continued a total of 15 days, with one trial per day, and commenced the day after the acclimation period ended. Each crayfish was placed in the start area (behind start line) at the base of the maze and given up to 30 minutes to complete the trial, i.e. locate the food reward. If the food was not found within this period of time the trial was ended and it was documented that that particular crayfish failed to find the food reward for that particular trial, and time to reward was recorded as 30 minutes so that a value was available for statistical analysis. An animal was deemed to have found the food reward once its rostrum crossed over the food, or once it touched the food with a

cheliped or antennae. If an animal succeeded in finding the reward the trial was ended at least ten minutes after the time to reward. Animals were given an extra ten minutes after food location in the T-maze so as to prevent them from associating locating the reward with being picked up and handled by the experimenter, which may have resulted in animals avoiding the food for this reason.

Crayfish were recorded as having crossed a landmark, i.e. under cover, start line, met junction line, or exit junction line once the tip of their rostrum met that particular landmark. However, an animal was still considered to be in the under cover area if its rostrum was sticking out while the rest of its body was covered. In initial experiments it was observed that oftentimes animals sat in this area of the maze with just their heads uncovered, thus, the need to vary the conditions by which an animal was considered under cover from those for meeting other maze landmarks (i.e. not based only on the rostrum). Therefore, an animal was considered under cover while oriented forward in the maze (head facing the junction) if roughly half of its body was covered, or at least up to the last set of legs. If oriented backwards in the maze (tail end was facing the junction), an animal was considered to be under cover as soon as its rostrum met the under cover area. The total amount of time each crayfish spent under cover was recorded at every trial.

Other recorded variables, in addition to time under cover and time to reward, included time needed for to perform ~180° turnarounds (a potential indicator of motor control) and time to reward arm (over repeated trials). As with time to reward, if an animal failed to locate the reward arm within the trial period the time to reward arm was recorded as 30 minutes. Each of these variables were compared between treatment groups and control animals to determine the

subthreshold toxic effects of atrazine, DEA, DIA, and HA on learning and behavior in *Procambarus clarkii*.

2.7. Statistical Analysis

Data were organized into Excel spreadsheets, and statistical analysis was performed using SAS (Statistical Analysis Software) 9.3. Repeated measures analysis of variance, ANOVA, was not appropriate for this data set as there were several instances of missing data, due to technical difficulties, and also because the data did not have a balanced design, i.e. not all treatment groups consisted of equal number of subjects. Procedure GLIMMIX (Bolker *et al*., 2008), which fits generalized linear mixed models by likelihood-based techniques and accommodates missing data and unbalanced designs, was used to model all data and perform analyses comparing each treatment group directly to control, as well as to perform pairwise comparisons between all treatments, for all response variables. Continuous time data for the following response variables: time to reward, time to reward arm, and time under cover were converted to decimal minutes. Percent of crayfish to not locate reward arm data were formatted as percentages, and time needed to complete ~ 180° turnarounds was recorded in seconds. All response variable data were uploaded into SAS and analyzed.

Procedure Univariate Normal Plot (Park, 2008) was used to test normality of all response variables' data, including time to reward, time to reward arm, percent of crayfish to not locate reward arm, time under cover, and ~ 180° turnaround times. Each response variable's data were found to be non-normally distributed, based on Shapiro-Wilk's p-value < .0001. Data for all response variables were overdispersed, even after fitted with Poisson distribution, and were thus modeled with a negative binomial distribution, which is commonly used for modeling outcome count variables that are highly overdispersed (UCLA, 2014)). The covariate structure was

specified as first order autoregressive, AR (1) in SAS, as repeated measurements on the same experimental unit are likely to be correlated and must be accounted for.

Analysis was primarily based on Solutions for Fixed Effects tables and Least Square Means of modeled data. Solutions tables estimate regression coefficients for each treatment compared to a reference level, which was set as control and equals 0, and show the directionality of each treatments' effects on a particular variable compared to control. The estimates (regression coefficients) are the approximate differences in mean response between each level and control, while the p-value tests the null hypothesis that the difference in the mean value from control equals 0, or that the confidence interval overlaps 0 (Frost, 2013). Least Square Means (LSMeans) are predicted population margins in which standard errors are adjusted for covariance parameters in the model. LSMeans may also be defined as linear combinations of the estimated means, or effects, that reflect the generalized mixed model being fit (Shafii & Price, 2014). LSMeans were especially useful as they allowed for pairwise comparisons between each treatment group.

The primary goal of the analysis was to determine if the treatment groups each differ significantly from control for several behavioral endpoints, which would indicate that exposure to individual, environmentally realistic concentrations of atrazine, DEA, DIA, or HA caused behavioral and learning impairments, thus indicating subthreshold toxicity. A secondary goal was to compare the various treatment groups, representing a range of concentrations for each chemical, to each other in order to determine the order of toxicity for contaminants and whether or not toxicity was greater at higher concentration.

Chapter 3. Results: Effects of Atrazine on Behavior

3.1. Justification of Data Fitting and Model Selection

Plots of raw, observed data means with confidence limits offer no information about the actual strength of the correlations of the data for within subject effects in a longitudinal study (High, 2011). Therefore, overlapping 95% confidence intervals for raw means can be inconclusive and misleading, as they must account for variation between subjects, and in the case of this study offered limited to no value for interpreting the actual significance of differences among the means of control and treatment groups of animals.

As explained in section 2.7, all response variables' data were modeled with a negative binomial distribution, which accounted for the extreme amount of overdispersion. Also, the covariate structures were specified as first order autoregressive, AR (1), to account for correlation that exists within longitudinal data within the same experimental units. The effect of treatment on each response variable was tested. Each of the models mentioned throughout the remainder of Chapter 3 had moderate to excellent model fit, based on Pearson Chi-Square / Degrees of Freedom (DF). Statistical analysis was based on tests of modeled data. As mentioned previously (Section 2.7), these tests included Solutions for Fixed Effects tables (which include regressions coefficients) and LSMeans comparing each treatment group to control, as well as to each other.

However, for some response variables (time to reward, time to reward arm and time under cover) the raw, observed data were also described for interpretation of trends that were observed over repeated trials. Such trends that occur over several days are not obvious based solely on regression coefficients and LSMeans. Thus, the longitudinal data presented are solely

descriptive pieces of data. The decision was made to not normalize these data, as this would decrease the obvious differences between control and atrazine treatment groups for these response variables and would deter from the overall message of figures displaying longitudinal data.

3.2. Location of Reward and Reward Arm

Each atrazine (ATR) treatment group initially took longer to locate the food reward (Figure 1.1) and the reward arm (Figure 1.2) than control during trial 1, a trend that continued throughout the course of the trial period, i.e. an additional 14 trials/days. The mean time to reward on trial day 1 for crayfish exposed to atrazine 3 µg/L was the nearest to control of all atrazine treatments, with a mean time of 9.87 minutes, versus 8.55 minutes for control. Thus, animals exposed to atrazine 3 µg/L, which was the closest performing atrazine treatment group to control (during trial 1) still required 15.44% longer than control to locate food reward initially. As stated previously, the difference between times to reward and to reward arm for control and atrazine treatment groups was immediate and continued throughout the course of the experiment. Therefore, crayfish exposed to atrazine had immediate impaired boldness to explore their environment compared to control, and familiarity with the environment didn't promote exploration of the maze by treated animals.

The model fit for time to reward data (Pearson Chi-Square / DF), was 0.99; thus, these data fit the model excellently. Model fit for time to reward arm data was also good with a chi-square / DF of 1.02. The type III tests of fixed effects of treatment resulted in a p-value of 0.0105 for time to reward data and a p-value = 0.0173 for time to reward arm data. Thus, treatment had a significant negative effect on animals' time to reward, as well as on time to reward arm.

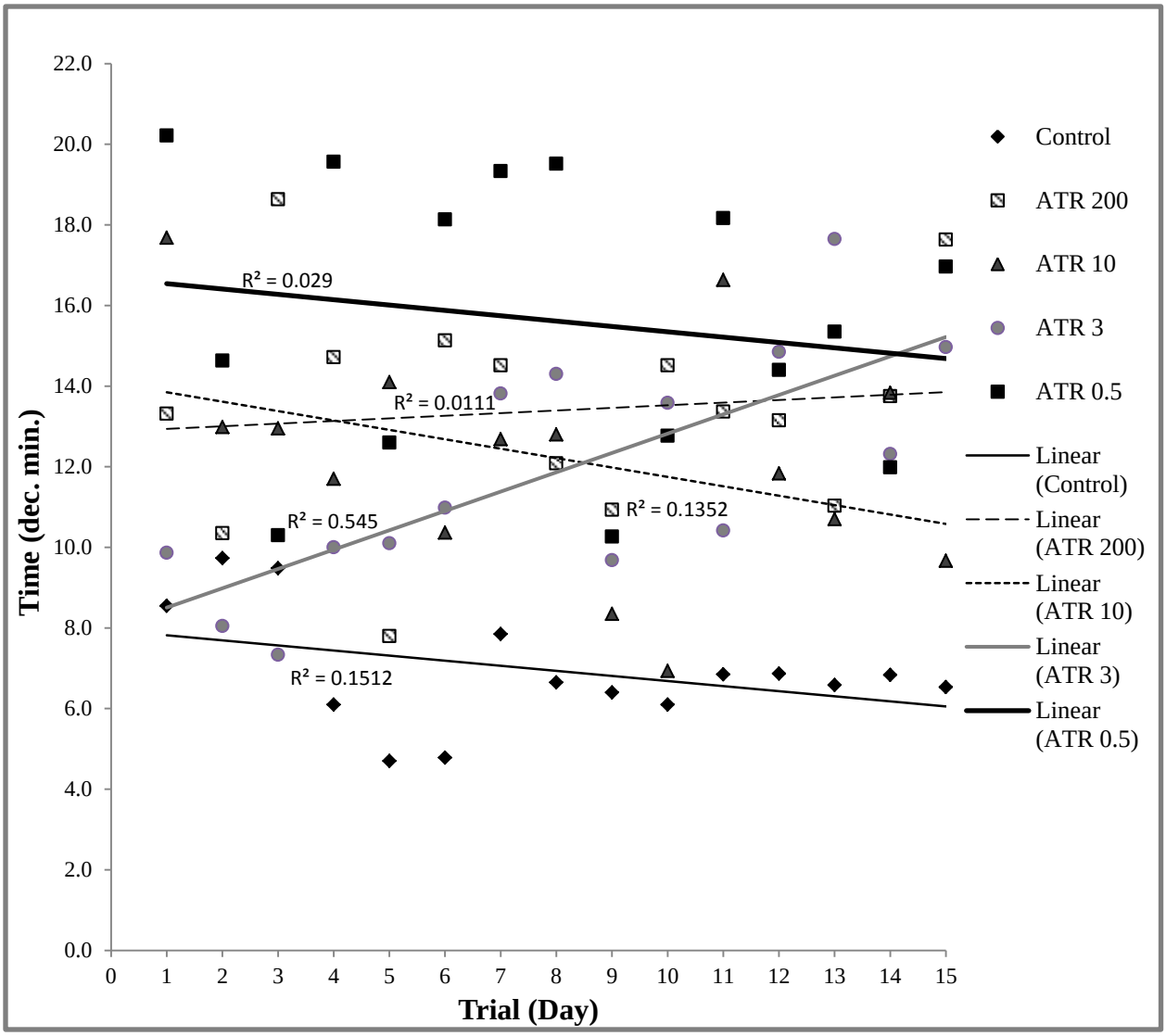

Fig. 3.1. Mean Time to Reward over Repeated Trials for control and atrazine treatment groups. Trendlines represent linear fit for data. Control: n = 15-17, ATR 0.5: n = 10-11, ATR 3: n = 10-12, ATR 10: n = 7-10, and ATR 200: n = 8-12.

3 µg/L treated crayfish performed the nearest to control on trial day 1, with a mean time of 9.87 minutes, versus 8.55 minutes for control. Therefore, the closest performing atrazine treatment group took 15.44% longer than control to initially locate the reward. Thus, atrazine treated crayfish exhibited immediate decreased boldness to explore compared to control, a trend that persisted throughout remaining trials

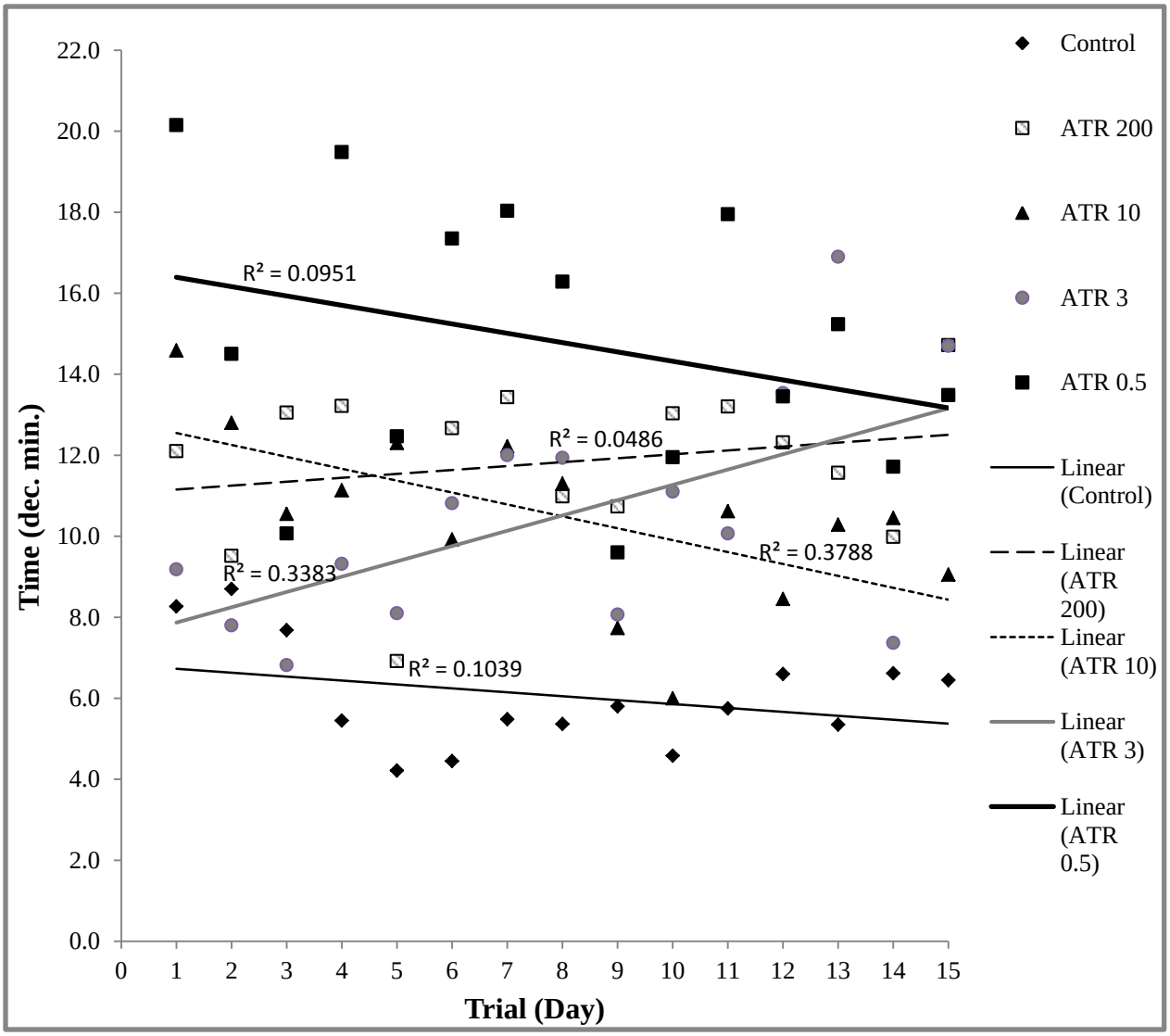

Fig. 3.2. Mean Time to Reward Arm over Repeated Trials for control and atrazine treatment groups. Trendlines represent linear fit for data. Control: n = 15-17, ATR 0.5: n = 10-11, ATR 3: n = 10-12, ATR 10: n = 7-10, and ATR 200: n = 8-12.

Treatment groups immediately took longer to find reward arm and continued do so for the remainder of the trial period. Crayfish treated with the lowest concentration of atrazine exhibited the greatest deficits in latency to reward arm.

A useful statistical tool in SAS for interpreting individual effects of treatment groups compared to control is Solutions for Fixed Effects, which provide regression coefficients (estimates) and p-values that allow one to determine if difference from control is significant and what the directionality of that difference is. Table 3.1 lists the estimated regression coefficients, standard error, p-value, alpha, and lower and upper confidence intervals for each atrazine treatment group for modeled time to reward data. Table 3.2 lists the Solutions for Fixed Effects data for modeled time to reward arm data. Asterisks (*) indicate significance from control.

Each atrazine treatment's regression coefficient was found to be significantly different from control ($p < 0.05$) for both time to reward and time to reward arm data. Furthermore, each estimate was positive, which indicates that atrazine at each concentration tested significantly increased mean time required for treated animals to locate the reward and the reward arm.

Results of LSMeans for modeled time to reward and time to reward arm data agree with results of Solutions tables 3.1 and 3.2. Each atrazine level tested took significantly longer than control to locate the reward (Figure 3.3). Interestingly, the lowest concentration of atrazine seemed to generate the greatest deficits in overall mean time to reward, while 200 µg/L atrazine was the least significantly different from control. Thus, at sublethal atrazine levels a nonlinear response was observed. Additionally, none of the atrazine treatment groups differed significantly from each other in time to reward LSMeans.

Each atrazine treatment group also took significantly longer to locate reward arm compared to control (Figure 3.4). Note that there was some variation between results for these two analyses; p-values for atrazine 3 µg/L and .05 µg/L were nearly identical for time to reward and time to reward arm, while the p-values for atrazine 200 µg/L and 10 µg/L differed more between these response variables. Regardless, for both analyses the results were the same

overall: each treatment level of atrazine was found to have significant behavioral deficits compared to control, indicating decreased boldness and willingness to explore the environment, even a familiar one, as a result of exposure to atrazine at various low doses.

In order to determine if there was evidence of learning and if length of time an animal was exposed to a particular concentration of atrazine may have influenced behavior, the effect of trial was tested at each treatment level. Model fits and p-values for type III fixed effects of trial for control and atrazine treatment groups for time to reward are listed in Table 3.3, while Table 3.4 lists this information for time to reward arm data.

Interestingly, there was no evidence of learning, i.e. a statistically significant effect of trial, in control crayfish neither for time to reward nor for time to reward arm. However, there was an effect of trial on animals exposed to 3 µg/L of atrazine for time to reward and time to reward arm. There was also an effect of trial on the atrazine 0.5 µg/L group for time to reward arm. It is noteworthy that this was the first analysis for which there was any real difference between results for time to reward and time to reward arm data. Analyses mentioned previously in this section have had the same atrazine treatment groups listed as significant for these two response variables, thus highlighting the importance of analyzing several behavioral endpoints when assessing toxicity.

There was a significant effect trial, or day, on time to reward, as well as time to reward arm, for crayfish exposed to 3 µg/L of atrazine. Referring again to Figure 3.1, one can see that as the length of time crayfish were exposed to atrazine 3 µg/L increased the mean time to reward generally increased, as well. Additionally, the same general trend was observed for this treatment group for longitudinal time to reward arm data. However, the data for time to reward arm were more scattered after trial 8 for atrazine 3 µg/L treatment group, with a time to reward arm on trial

day 14 that was less than time on trial one. However, explaining every single variation in results is difficult, if not impossible, as well as unwise. Regardless, in general the longer animals were exposed to this treatment the more pronounced the observed deficits in time to reward and time to reward arm. The same cannot be inferred for atrazine 0.5 µg/L data; however, although, there is statistically a significant effect of trial on time to reward arm. Atrazine 0.5 µg/L time to reward arm data are chaotic and scattered, making interpretation of any sort of a pattern impossible. One may infer that evidence of such chaotic data implies that animals exhibited more erratic behavior as a result of toxicant exposure, which supports the notion that animals were sick due to atrazine exposure.

Table 3.1. Time to Reward Solutions for Fixed Effects. Each atrazine treatment took longer to locate reward (based on positive estimate) and these differences were significantly greater than control (based on $p < 0.05$), indicated by *. Alpha = 0.05.

Treatment	Estimate	Standard Error	p	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
ATR 0.5 (n = 10 -11)	0.8108	0.2152	0.0002 *	0.3886	1.2329
ATR 3 (n = 10-12)	0.5896	0.21	0.005 *	0.1777	1.0015
ATR 10 (n = 7-10)	0.5759	0.2221	0.0096 *	0.1403	1.0115
ATR 200 (n = 8-12)	0.4755	0.2114	0.0246 *	0.06087	0.8901

Table 3.2. Time to Reward Arm Solutions for Fixed Effects. Every atrazine treatment took significantly longer to locate the reward arm compared to untreated crayfish, indicated by *. Alpha = 0.05.

Treatment	Estimate	Standard Error	p	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
ATR 0.5 (n = 10 -11)	0.8998	0.2378	0.0002 *	0.4333	1.3662
ATR 3 (n = 10-12)	0.6423	0.2320	0.0057 *	0.1872	1.0973
ATR 10 (n = 7-10)	0.5408	0.2455	0.0277 *	0.05928	1.0223
ATR 200 (n = 8-12)	0.4733	0.2336	0.0429 *	0.01518	0.9314

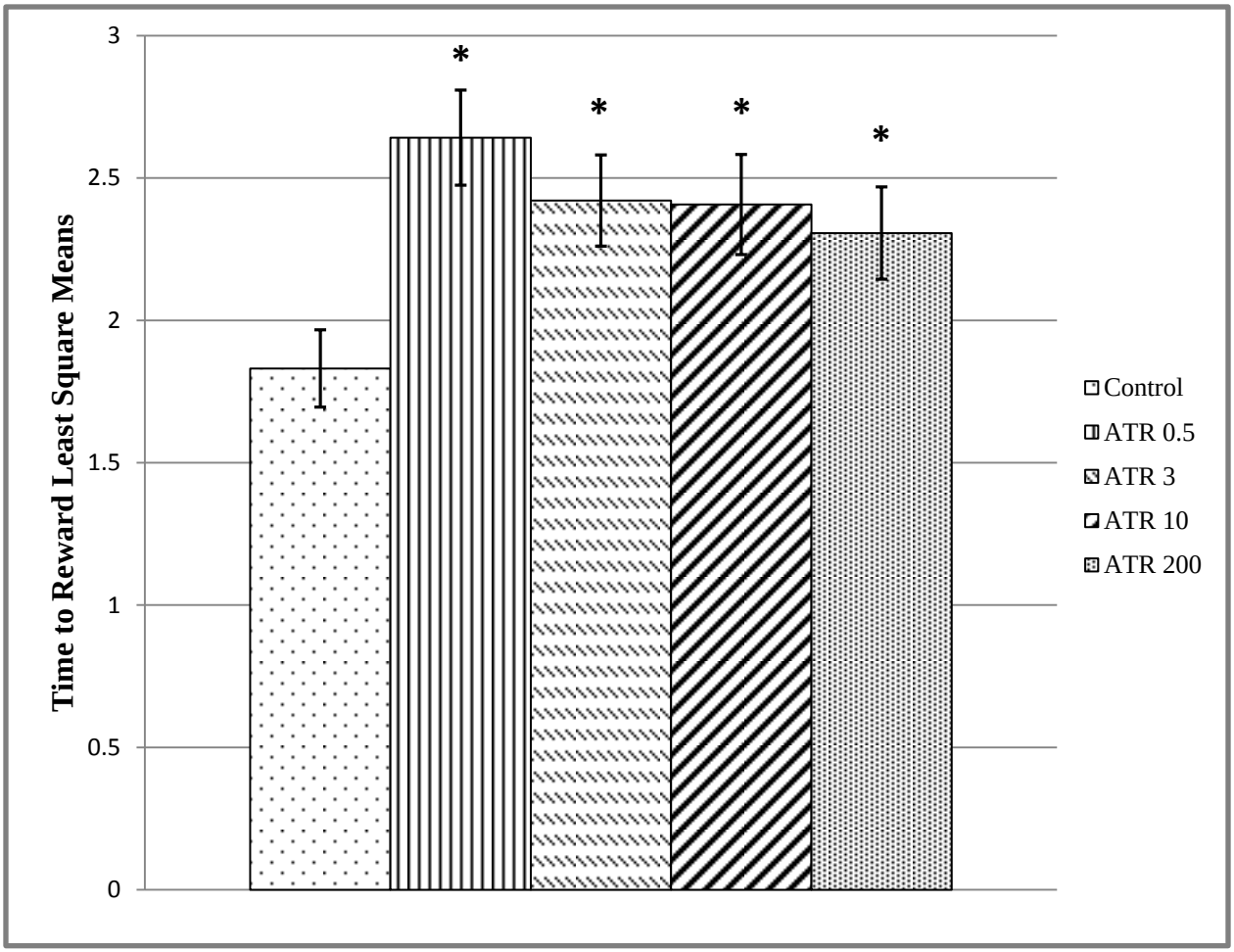

Fig. 3.3. Time to Reward LSMeans for control and atrazine treatment groups. * indicates a statistically significant difference from control (n = 15-17), or $p < 0.05$, and error bars represent 95% confidence intervals.

- ATR 0.5 (n = 10-11): p = 0.0002
- ATR 3 (n = 10-12): p = 0.005
- ATR 10 (n = 7-10): p = 0.0096
- ATR 200 (n = 8-12): p = 0.0246.

ATR 0.5 µg/L treated crayfish had the greatest latency to reward, while the higher atrazine treatment groups, although still significant from control, took less time to locate reward than did ATR 0.5 group.

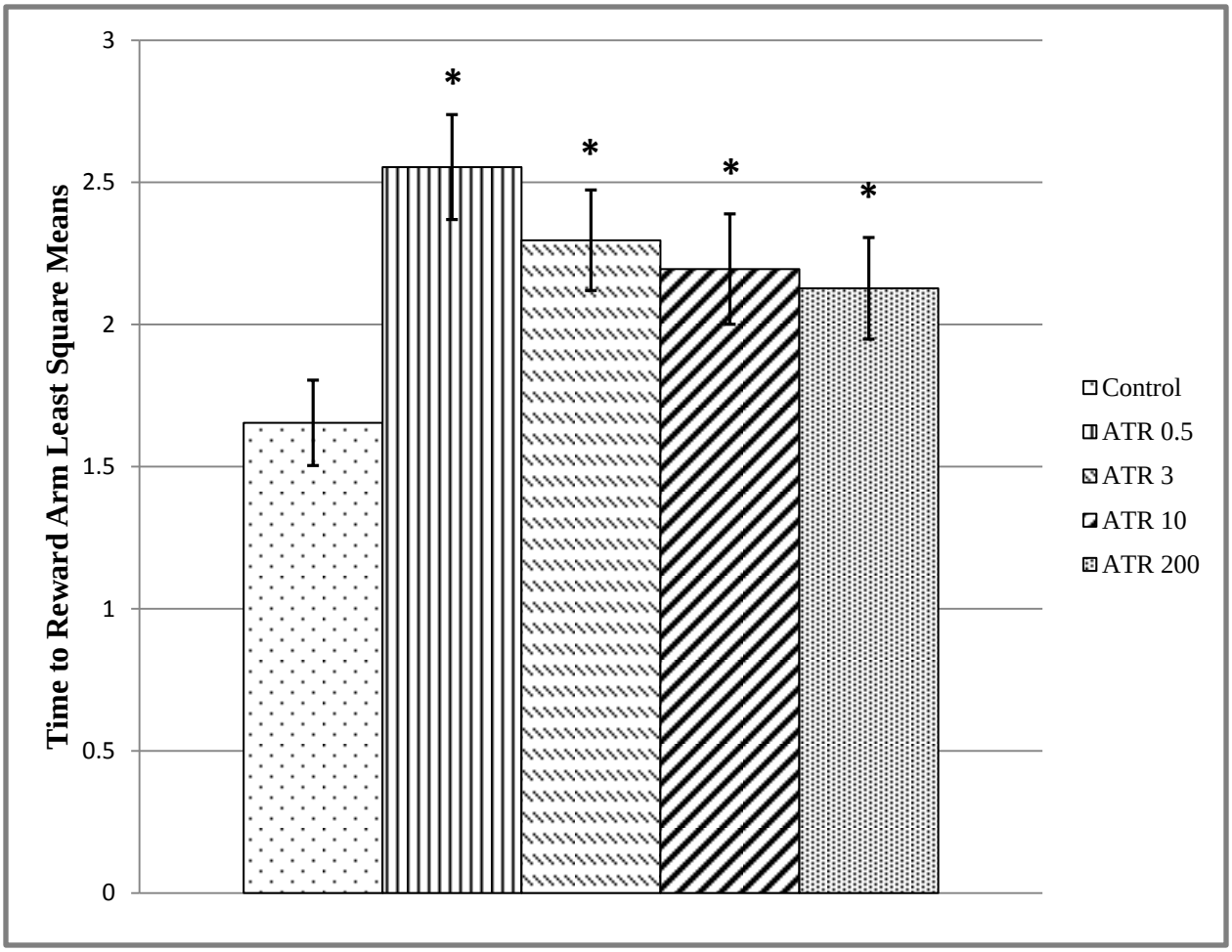

Fig. 3.4. Time to reward arm LSMeans for control and atrazine treatment groups. * indicates a statistically significant difference from control (n = 15-17), and error bars represent 95% confidence intervals.

- ATR .05 (n = 10-11): p = 0.0002
- ATR 3 (n = 10-12): p = 0.0057
- ATR 10 (n = 7-10): p = 0.0277
- ATR 200 (n = 8-12): p = 0.0429

Results of time to reward arm are similar to those of time to reward; the lowest atrazine treatment group exhibited the most significant deficit in latency to reward arm, and all atrazine treatments took significantly longer than control to locate reward arm.

Table 3.3. Effect of Trial by Treatment for Time to Reward. ATR 3 µg/L treated animals had a significant effect of trial on latency to reward (indicated by *)

Treatment	**Fit: Chi-square / DF**	**p-value**
Control (n = 15-17)	1.07	0.0610
ATR 0.5 (n = 10 -11)	0.85	0.1996
ATR 3 (n = 10-12)	0.97	0.0005 *
ATR 10 (n = 7-10)	0.81	0.6479
ATR 200 (n = 8-12)	0.99	0.3163

Table 3.4. Effect of Trial by Treatment for Time to Reward Arm. ATR 3 and ATR 0.5 µg/L treated animals had a significant effect of trial on latency to reward arm, as indicated by *. This is the first test in which time to reward arm data do not mirror results of time to reward data.

Treatment	**Fit: Chi-square / DF**	**p-value**
Control (n = 15-17)	1.14	0.1014
ATR 0.5 (n = 10 -11)	0.89	0.0315 *
ATR 3 (n = 10-12)	0.97	0.0029 *
ATR 10 (n = 7-10)	0.91	0.5551
ATR 200 (n = 8-12)	0.94	0.7712

3.3. Percent of Crayfish to Not Locate Reward Arm

The model fit for percent of crayfish to not locate reward arm, chi-square / DF, was marginal at 0.73. The p-value for treatment (Type III Tests) was < .0001. In order to determine the individual effects of treatments on this variable solutions for fixed effects and LSMeans tests were performed.

The results for percent of crayfish to not locate the reward arm within the trial period (30 minutes) offered greater variation than observed thus far for other response variables. All atrazine treatment groups' estimates, or regression coefficients, were positive, thus indicating that each treatment group had a greater overall percentage of crayfish that did not find the reward arm compared to control (Table 3.5). However, this difference was not significant from control for the atrazine 3 µg/L treatment group, as indicated by $p > 0.05$ and a confidence interval that overlapped 0.

In Figure 3.5, one can easily see that there were several significant differences between treatment groups, as well. LSMeans of atrazine 0.5, 10 µg/L, and 200 µg/L were found to be significantly greater than that of atrazine 3 µg/L, and therefore these treatments groups had significantly more animals fail to locate the reward arm compared not only to control, but also to atrazine 3 µg/L.

Recall, however, that there was a significant increase in time to reward arm for the atrazine 3 µg/L treatment group (Fig. 3.4). Therefore, although percentage-wise there was no difference in success rates to find reward arm between control and ATR 3, for those animals that did locate the reward arm it took significantly longer compared to control. These somewhat contradictory results further support the need for multiple behavioral analyses when attempting

to determine the potential toxicity of environmental contaminants. Additionally, these results further support a nonlinear toxicity response to atrazine concentration, which was suggested by previous analyses, as well.

Table 3.5. Percent Crayfish to Not Locate Reward Arm Solutions for Fixed Effects. All atrazine treatment groups, excluding ATR 3, had significantly more crayfish not locate reward arm. * Indicates a significant difference from control. Alpha = 0.05.

Treatment	Estimate	Standard Error	p	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
ATR 0.5 (n = 10 -11)	1.8383	0.3963	<.0001 *	1.0555	2.6211
ATR 3 (n = 10-12)	0.7459	0.403	0.0661	-0.0503	1.5421
ATR 10 (n = 7-10)	1.2383	0.3991	0.0023 *	0.4499	2.0267
ATR 200 (n = 8-12)	1.676	0.3969	0.0429 *	0.05	0.892

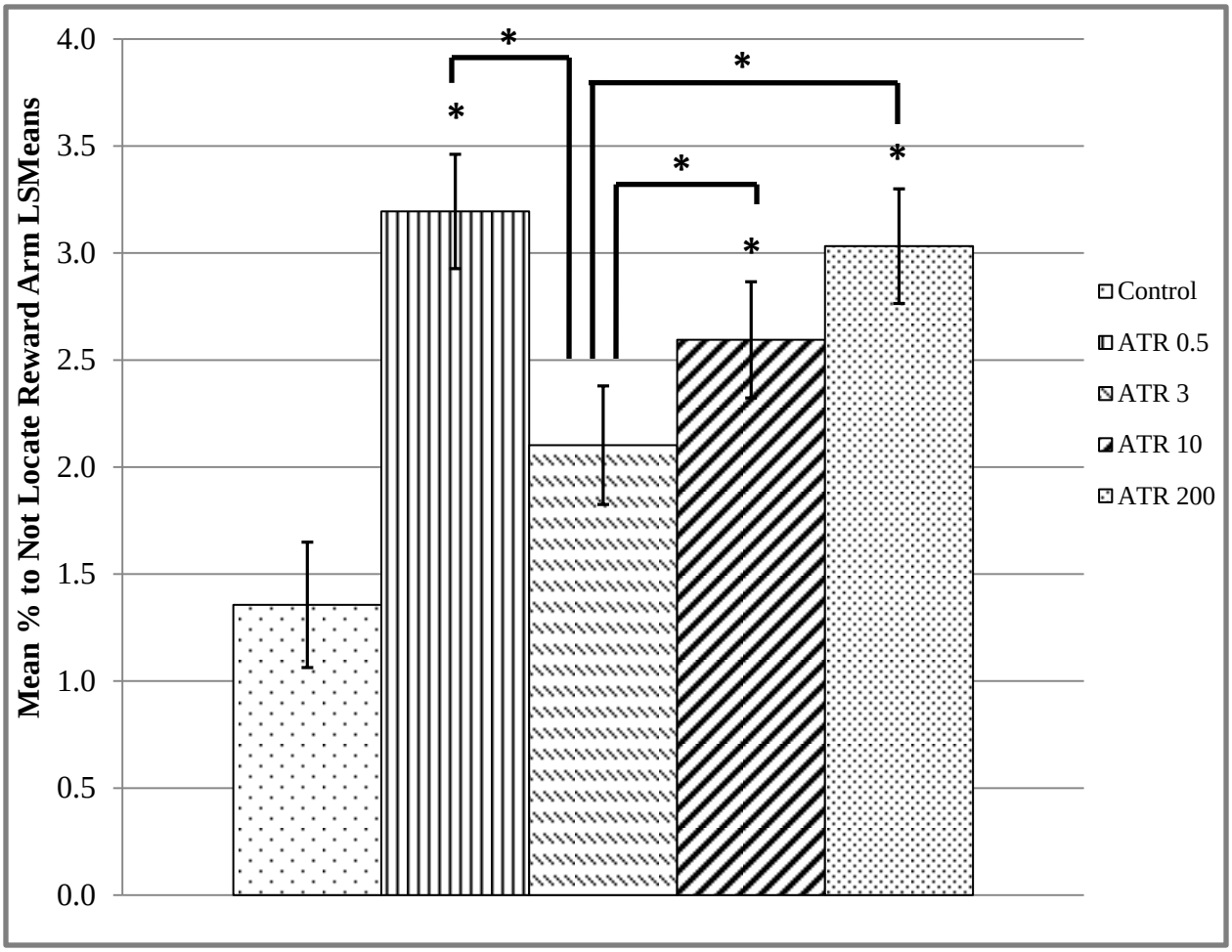

Fig. 3.5. Mean Percent of Crayfish to Not Locate Reward Arm LSMeans. * directly above error bars represent significant difference from control (n = 15-17), and error bars represent 95% confidence intervals.

- ATR 0.5 (n = 10-11): p <.0001
- ATR 3 (n = 10-12): p =.0661
- ATR 10 (n = 7-10) p =.0023
- ATR 200 (n = 8-12) p <.0001)

* above horizontal bars represent significant differences between treatment groups. ATR 3 µg/L had significantly less crayfish locate reward arm than other ATR treatment groups. ATR 3 uµg/L was not significantly different from control.

3.4. Time Spent in Under Cover Area of the T-Maze

The model fit for under cover data, chi-square / DF, was 1.04. The p-value for treatment (Type III Tests) was < .0001; therefore, there was a very significant effect of treatment on time spent in under cover area. In order to determine the individual effects of treatments on time spent under cover solutions for fixed effects and LSMeans tests were performed.

Based on regression coefficients, as well as p-values, (Table 3.6) crayfish exposed to each level of atrazine treatment spent significantly longer than control in the under cover area of the maze, excluding the highest treatment group (ATR 200). Atrazine 0.5 µg/L treated crayfish had the most significant difference from control, with a p value <.0001, followed by atrazine 10 µg/L and atrazine 3 µg/L. This is the first analysis in which results for atrazine 200 µg/L were not significantly different from control. However, each atrazine treatment had a positive estimate value, including, atrazine 200 µg/L. Thus, all treatment groups spent more time under cover, but the difference from control was only significant for animals exposed to the three lowest levels of atrazine.

Furthermore, based on under cover LSMeans (Figure 3.6) of control and atrazine treatment groups, one can easily see which treatments different significantly from control, as well as from each other. Crayfish exposed to atrazine 200 µg/L spent significantly less time under cover than did atrazine 0.5 µg/L and atrazine 10 µg/L groups. Note that there was not a significant difference between atrazine 200 µg/L and atrazine 3 µg/L. These results indicate, once again, a nonlinear toxic response to atrazine concentration at the sublethal level. Additionally, these results suggest that exposure of crayfish to atrazine at 0.5, 3, and 10 µg/L causes impairments in explorative behavior and increased lethargy.

Table 3.6. Under Cover Solutions for Fixed Effects. Animals exposed to the three lowest atrazine concentrations spent significantly more time than control under cover. * Indicates significant difference from control. Crayfish treated with 200 µg/L of atrazine spent more time under cover, based on a positive regression coefficient, than control, but this difference was not significant ($p \geq 0.05$). Alpha = 0.05.

Treatment	Estimate	Standard Error	p	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
ATR 0.5 (n = 10 -11)	1.9733	0.3971	<.0001 *	1.1944	2.7522
ATR 3 (n = 10-12)	1.2237	0.3883	0.0017 *	0.4621	1.9853
ATR 10 (n = 7-10)	1.5897	0.4094	0.0001 *	0.7868	2.3927
ATR 200 (n = 8-12)	0.689	0.3933	0.08	-0.0824	1.4604

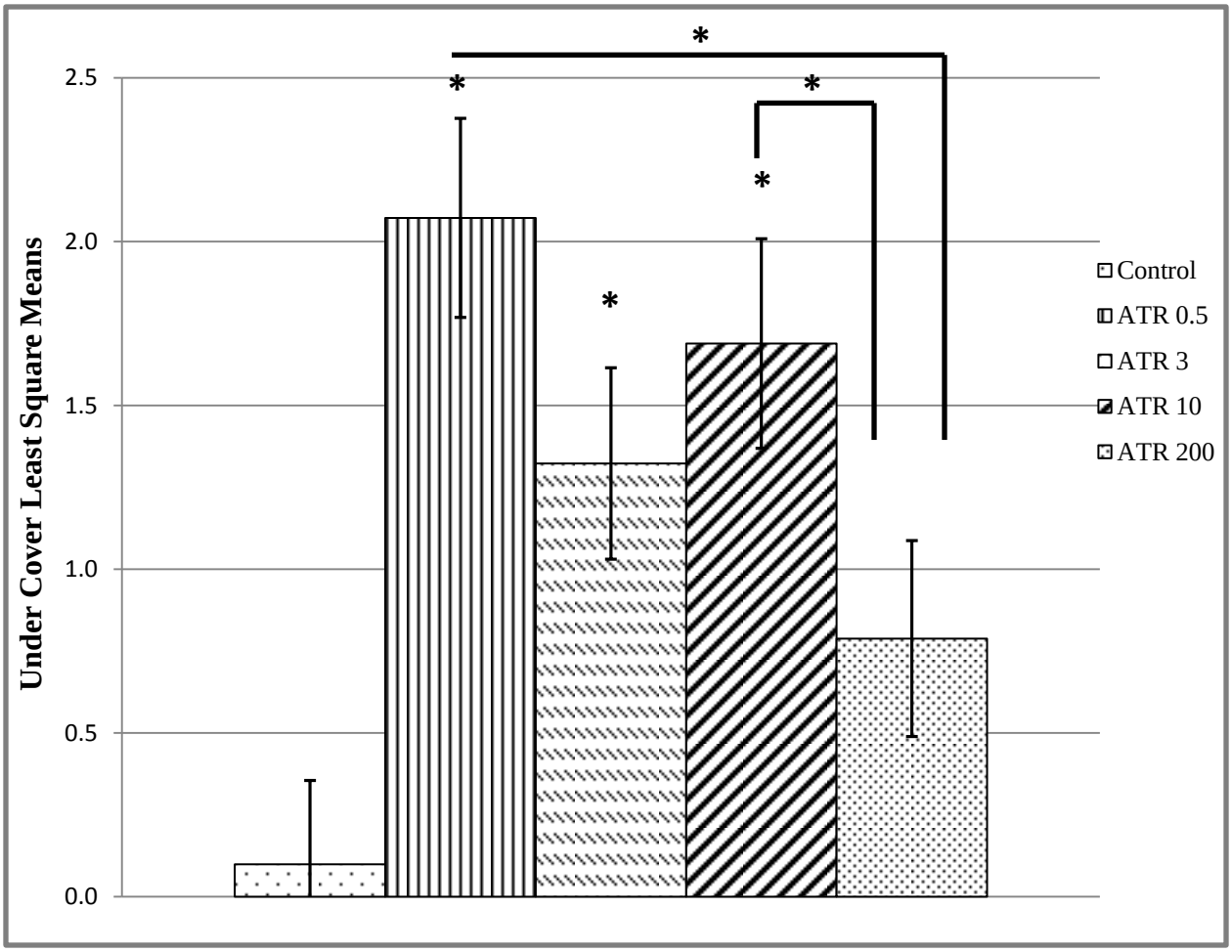

Fig. 3.6. Under Cover Least Square Means. * directly above error bar represents significant difference from control (n = 15-17). Error bars represent 95% confidence intervals.

- ATR 0.5 (n = 10-11): $p < .0001$
- ATR 3 (n = 10-12): $p = 0.0017$
- ATR 10 (n = 7-10): $p = 0.0001$
- ATR 200 (n = 8-12): $p = 0.08$)

* above horizontal lines represents significant difference between treatment groups. ATR 200 µg/L treated animals spent significantly less time under cover than ATR 10 & ATR 0.5. There was not a significant difference between ATR 3 and ATR 200.

Effect of trial was tested for all treatment groups, including control, in order to determine if length of time animals were exposed to various levels of atrazine may have had an effect on time spent under cover and/or if familiarity with the maze, which would presumably increase with repeated trials, influenced maze performance for controls and atrazine treatment groups. Table 3.7 lists the model fit for each treatment, in which effect was trial and time under cover was response, as well each models' respective p-values (type III tests).

Table 3.7. Effect of Trial on Under Cover Time. There was a significant effect of trial on time spent under cover by control group, as indicated by *. Trial did not prove to have a significant effect on under cover time for any atrazine treatment groups.

Treatment	**Fit: Chi-square / DF**	**p-value**
Control (n = 15-17)	1.02	0.0004 *
ATR 0.5 (n = 10 -11)	0.74	0.0927
ATR 3 (n = 10-12)	1.04	0.3570
ATR 10 (n = 7-10)	0.86	0.8113
ATR 200 (n = 8-12)	1.10	0.5215

The only group for which trial had a significant effect on time spent under cover was control (Table 3.7). For each of the atrazine treatments there was not a significant effect of trial, or $p > 0.05$. It is helpful to view the data in conjunction with Figure 3.7, which contains raw, observed data of mean time under cover over repeated trial for control and atrazine treatment groups. In Figure 3.7 one can see that for control animals as trial/day progressed the mean time spent under cover decreased in general, and as there was a significant effect of trial one may infer that the observed decrease in time spent under cover over repeated trials was significant. Therefore, control crayfish became more explorative and bold as they became more familiar with their environment, while atrazine treated crayfish did not. Furthermore, one may also extend this interpretation to infer that perhaps this is evidence of learning in control crayfish. In effect, it is plausible that as untreated animals became more familiar with their environment and learned that it was not threatening (lacked potential predators) they became more willing to explore said environment and spent less time at the base of the maze in the under cover area. Thus, crayfish exposed to atrazine 0.5, 3, and 10 µg/L exhibited significant deficits, compared to control and spent more time under cover, an indication of toxicant induced deficits in explorative behavior and boldness, as well as increased lethargy, which perhaps may also suggest that these particular treatment groups also had impairments in learning.

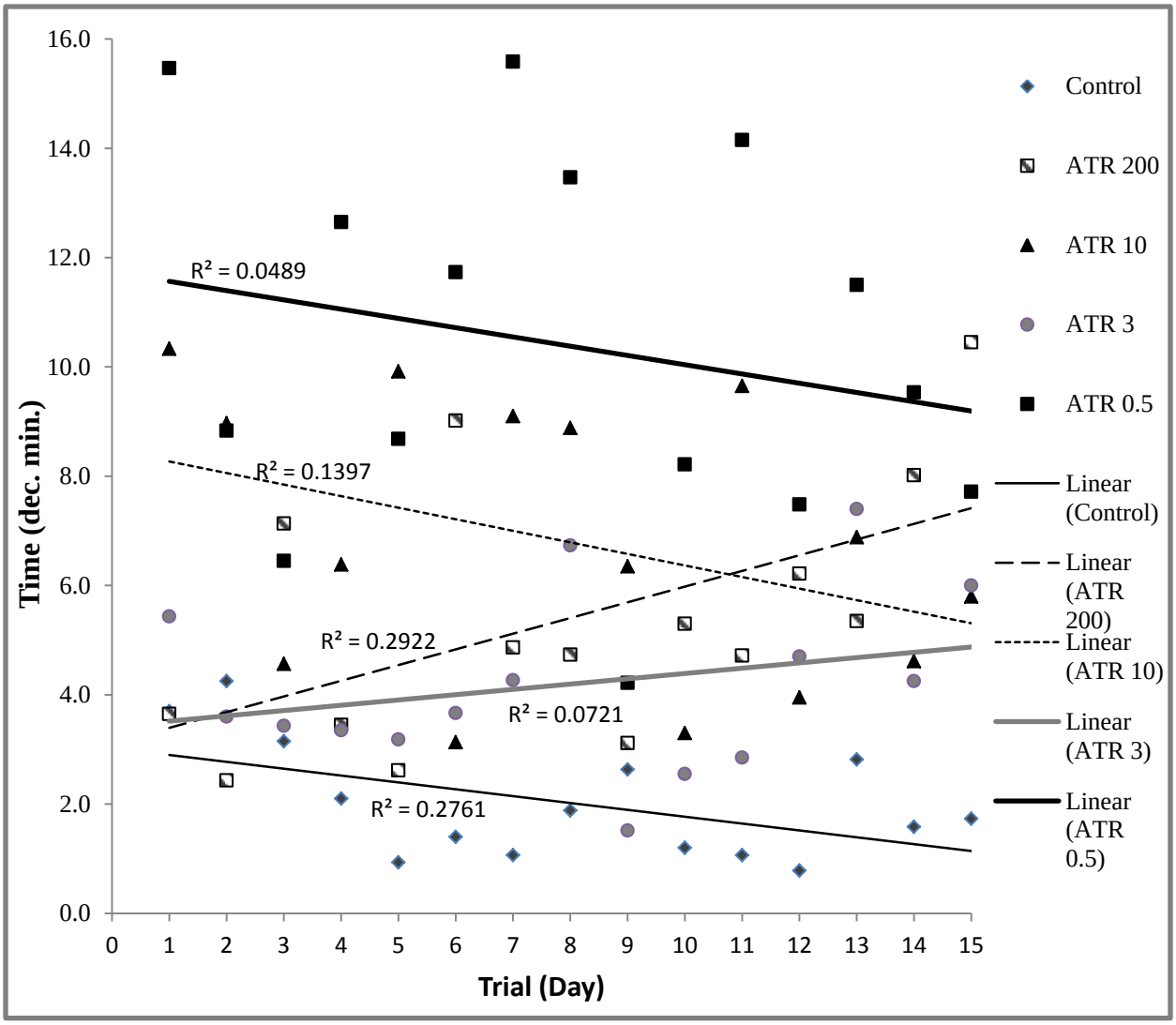

Fig. 3.7. Mean time under cover over repeated trials for control and atrazine treatment groups. Trendlines represent linear fit for data. Control: n = 15-17, ATR 0.5: n = 10-11, ATR 3: n = 10-12, ATR 10: n = 7-10, and ATR 200: n = 8-12. There was a significant effect of trial (day) on time spent under cover for untreated crayfish. Therefore, control group spent significantly less time, at least until trial 12, under cover as trials progressed; thus, control animals became significantly more bold and explorative as they became more familiar with the maze. Additionally, one may infer that this is evidence of learning in control crayfish. Atrazine treated crayfish, excluding ATR 200, spent significantly more time under cover than control (Fig. 3.6); thus, crayfish exposed to the three lowest concentrations of atrazine had impaired boldness and elevated lethargy compared to controls.

3.5. Time Needed to Complete ~ 180° Turnarounds

The model fit for ~ 180° turnaround data, chi-square / DF, was marginal at 0.89, and the p-value for treatment (Type III Tests) was < .0001. Thus, treatment had a very significant effect on the time needed for crayfish to complete roughly 180° turnarounds. Compared to control every atrazine treatment group of crayfish took longer to complete turnarounds, as indicated by positive regression coefficients (estimate values in Table 3.8). Furthermore, these elevated values were significantly different from control for each atrazine treatment group. Refer to table 3.8 for modeled turnaround data's solutions for fixed effects values.

The predicted population margins results (in which standard errors are adjusted for covariance parameters in the model), or LSMeans, echoed those of the regression coefficients for turnaround data. Each treatment group took significantly longer to perform ~ 180° turnarounds compared to control (Figure 3.8). Assuming ~ 180° turnaround times may be used to interpret motor control, these results suggest that exposure to subthreshold levels of atrazine resulted in deficits in crayfish motor response.

Table 3.8. ~ 180° Turnaround Solutions for Fixed Effects. Each atrazine treatment group took significantly longer than control (indicated by *) to perform turnarounds compared to control, an indication of impaired motor control in treated crayfish. Alpha = 0.05.

Treatment	Estimate	Standard Error	P	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
ATR 0.5 (n = 10 -11)	0.6241	0.1078	<.0001 *	0.4126	0.8356
ATR 3 (n = 10-12)	0.4766	0.1012	<.0001 *	0.2780	0.6752
ATR 10 (n = 7-10)	0.5874	0.1118	<.0001 *	0.3679	0.8069
ATR 200 (n = 8-12)	0.4094	0.1004	<.0001 *	0.2123	0.6064

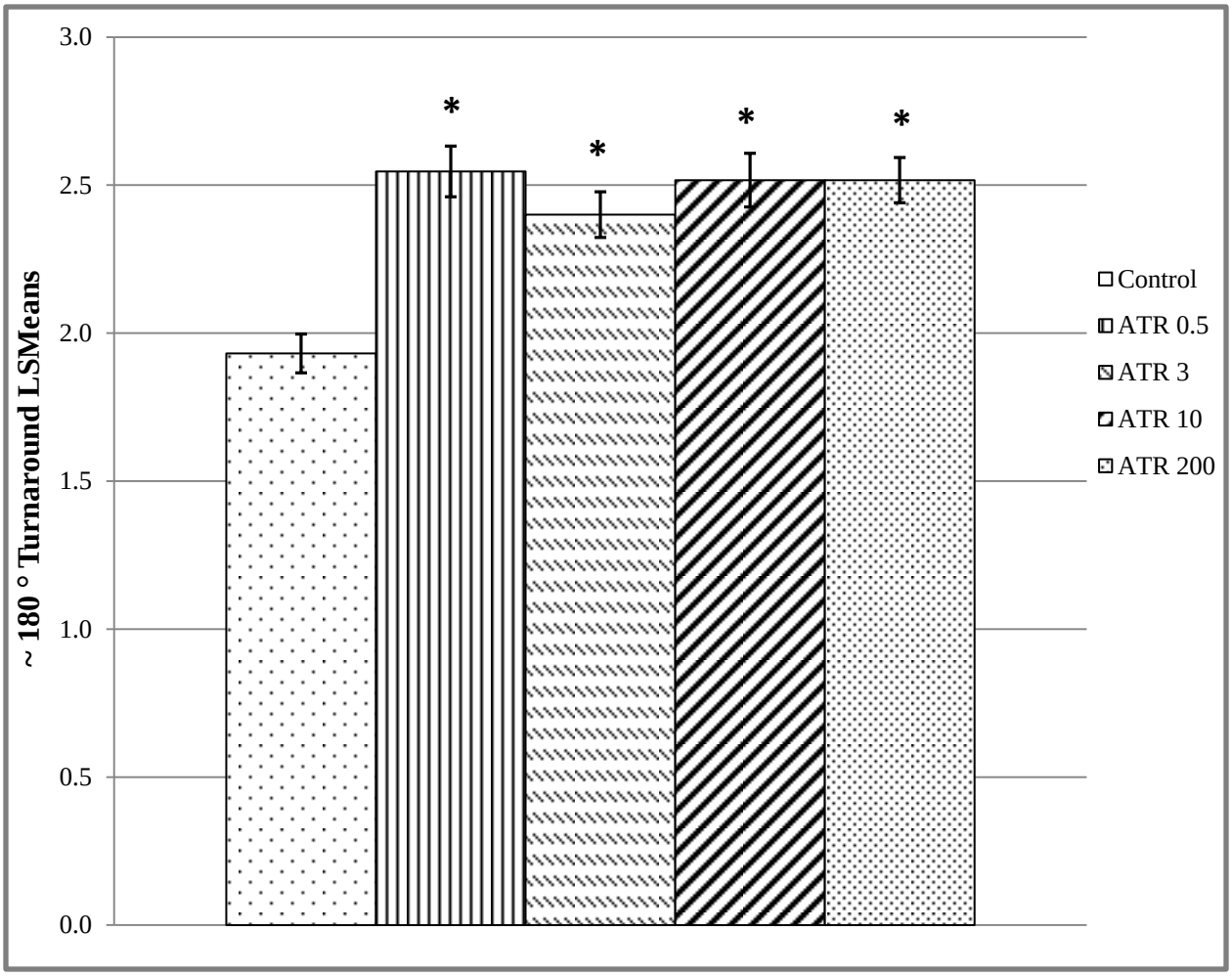

Fig. 3.8. ~ 180° Turnaround Least Square Means. * Indicates significance from control (n = 15-17), and error bars represent 95% confidence intervals. P <.0001 for each atrazine treatment group compard to control (n = 15-17). Sample sizes for atrazine treatment groups were as follows: ATR 0.5 (n = 10-11), ATR 3 (n = 10-12), ATR 10 (n = 7-10), and ATR 200 (n = 8-12).

P-values for each atrazine treatment group <.0001; thus, each atrazine treatment group took significantly longer to perform turnarounds than control. Additionally, there was no significant difference in turnaround times between any atrazine treatment groups.

Chapter 4. Results: Effects of Atrazine Degradates on Behavior

4.1. Justification of Data Fitting and Model Selection

As explained in section 3.1, plots of raw, observed data means were not used for statistical analysis, as they offer no information about the actual strength of the correlations of the data for within subject effects in a longitudinal study (High, 2011). All response variables' data were modeled with a negative binomial distribution (accounting for extreme overdispersion), and the covariate structures were specified as first order autoregressive to account for correlation of longitudinal data. The effect of treatment on each response variable was tested. Each of the models mentioned throughout the remainder of Chapter 4 had moderate to excellent model fit (Pearson Chi-Square / DF). Statistical analysis was based on tests of modeled data, including Solutions for Fixed Effects tables (which include regressions coefficients) and LSMeans, which allowed pairwise comparisons between degradate treatment groups as well as to atrazine treatment groups.

Similarly to Chapter 3, some response variables' (time to reward, time to reward arm and time under cover) raw, observed data are also described for interpretation of trends that were observed over repeated trials. Thus, the longitudinal data presented are solely descriptive pieces of data. The decision was made to not normalize these data, as this would decrease the obvious differences between control and atrazine treatment groups for these response variables and would deter from the overall message of figures displaying longitudinal data.

4.2. Location of Reward & Reward Arm

Effect of treatment was tested for modeled time to reward and time to reward arm data. All treatment data were modeled together; thus, the model fits are the same as mentioned previously in Section 3.1. Model fit for time to reward data was 0.99, and model fit for time to reward arm data was 1.02. The type III tests of fixed effects were as follows: $p = 0.0105$ for time to reward data and $p = 0.0173$ for time to reward arm data. Therefore, treatment had a significant effect on time to reward, as well as time to reward arm.

The regression coefficients for atrazine's degradates were similar for modeled time to reward, Table 4.1, and time to reward arm data, Table 4.2. All estimates were positive for both response variables for DEA, DIA, and HA, indicating that animals exposed to these chemicals took longer to find the food reward and locate the reward arm compared to control crayfish. However, DIA 0.5 µg/L and DIA 10 µg/L were not significantly different from control, as indicated by $p > 0.05$, while crayfish exposed to DEA and HA (at both concentrations) took significantly longer for both response variables.

Table 4.1. Time to Reward Solutions for Fixed Effects. DEA and HA treatment groups had significantly greater latency to reward compared to control, indiacted by *. Alpha = 0.05.

Treatment	Estimate	Standard Error	P	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
DEA 0.5 (n = 6-11)	0.4528	0.2159	0.0361 *	0.02937	0.8763
DEA 10 (n = 7-11)	0.5212	0.2161	0.0160 *	0.09730	0.9451
DIA 0.5 (n = 5-11)	0.3045	0.2165	0.1598	-0.1202	0.7292
DIA 10 (n = 8-11)	0.3566	0.2160	0.0990	-0.06709	0.7803
HA 0.5 (n = 6-11)	0.7149	0.2155	0.0009 *	0.2921	1.1376
HA 10 (n = 9-11)	0.7347	0.2154	0.0007 *	0.3122	1.1573

Table 4.2. Time to Reward Arm Solutions for Fixed Effects. DEA and HA treated crayfish too significantly longer to locate the reward arm compared to control, indicated by *. Alpha = 0.05. Results agree with those presented in Table 4.1.

Treatment	Estimate	Standard Error	P	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
DEA 0.5 (n = 6-11)	0.4709	0.2385	0.0485 *	0.003173	0.9386
DEA 10 (n = 7-11)	0.6441	0.2387	0.0070 *	0.1759	1.1122
DIA 0.5 (n = 5-11)	0.3874	0.2391	0.1053	-0.08145	0.8563
DIA 10 (n = 8-11)	0.4042	0.2386	0.0905	-0.06389	0.8722
HA 0.5 (n = 6-11)	0.7593	0.2382	0.0015 *	0.2922	1.2264
HA 10 (n = 9-11)	0.7682	0.2381	0.0013 *	0.3011	1.2352

Degradate regression coefficients are supported by results obtained from LSMeans tests of modeled time to reward, Figure 4.1, and time to reward arm data, Figure 4.2. Significant differences ($p < 0.05$) from control are indicated by asterisks above error bars. Although DIA 0.5 µg/L and DIA 10 µg/L were not significantly different from control for time to reward or time to reward arm, neither DIA treatment was significantly different from DEA or HA treatments (for time to reward or time to reward arm). Additionally, it was found that ATR 0.5 µg/L treated crayfish took significantly longer to locate the reward than the DIA 0.5 µg/L group ($p = 0.0331$). Pairwise comparisons of LSMeans between treatment groups suggest that DIA is slightly less toxic than DEA and HA (but not significantly so), and that it is also significantly less toxic than ATR (for time to reward), but only at the lowest concentrations tested (refer to tables and figures in Appendix). There was not a significant difference between DIA 10 µg/L and any ATR group. Also, the three highest ATR treatment groups were not found to be significantly different from degradate treatment groups for time to reward or time to reward arm. However, as stated previously, analysis of different behavioral endpoints often leads to varying results; thus, it is wise to avoid making judgments on chemical toxicity based solely on a single analysis.

Effect of trial was tested for all degradate treatment groups to determine if length of time animals were exposed to 0.5 µg/L and 10 µg/L of DEA, DIA or HA may have had an effect on time to reward and time to reward arm. Table 4.3 lists the model fit for each degradate treatment, in which trial was the effect and time to reward was the response, as well each models' respective p-values (type III tests), and Table 4.4 lists the same information, but for response variable time to reward arm.

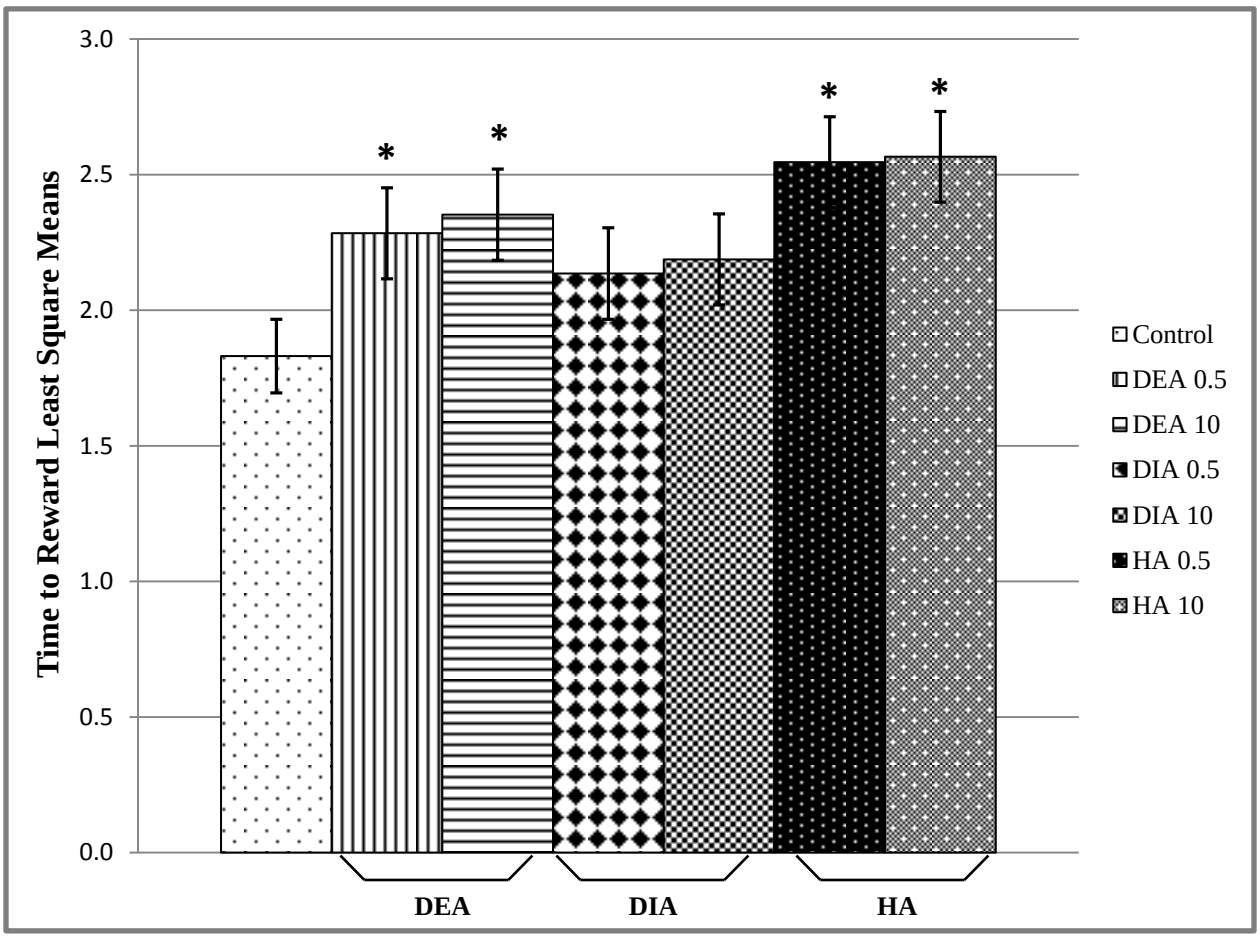

Figure 4.1. Time to reward LSMeans. * Indicate significance from control (n = 15-17), and error bars represent 95% confidence intervals.

- DEA 0.5 (n = 6-11): p = 0.0361
- DEA 10 (n = 7-11): p = 0.016
- DIA 0.5 (n = 5-11): p = 0.1598
- DIA 10 (n = 8-11): p = 0.099
- HA 0.5 (n = 6-11): p = 0.0009
- HA 10 (n = 9-11): p = 0.0007

DIA treatments were not significantly different from control, and none of the degradate treatments were significantly different from each other, indicating that DIA is the least toxic of the degradation products tested. DEA and HA treatment groups took significantly longer than control to locate reward.

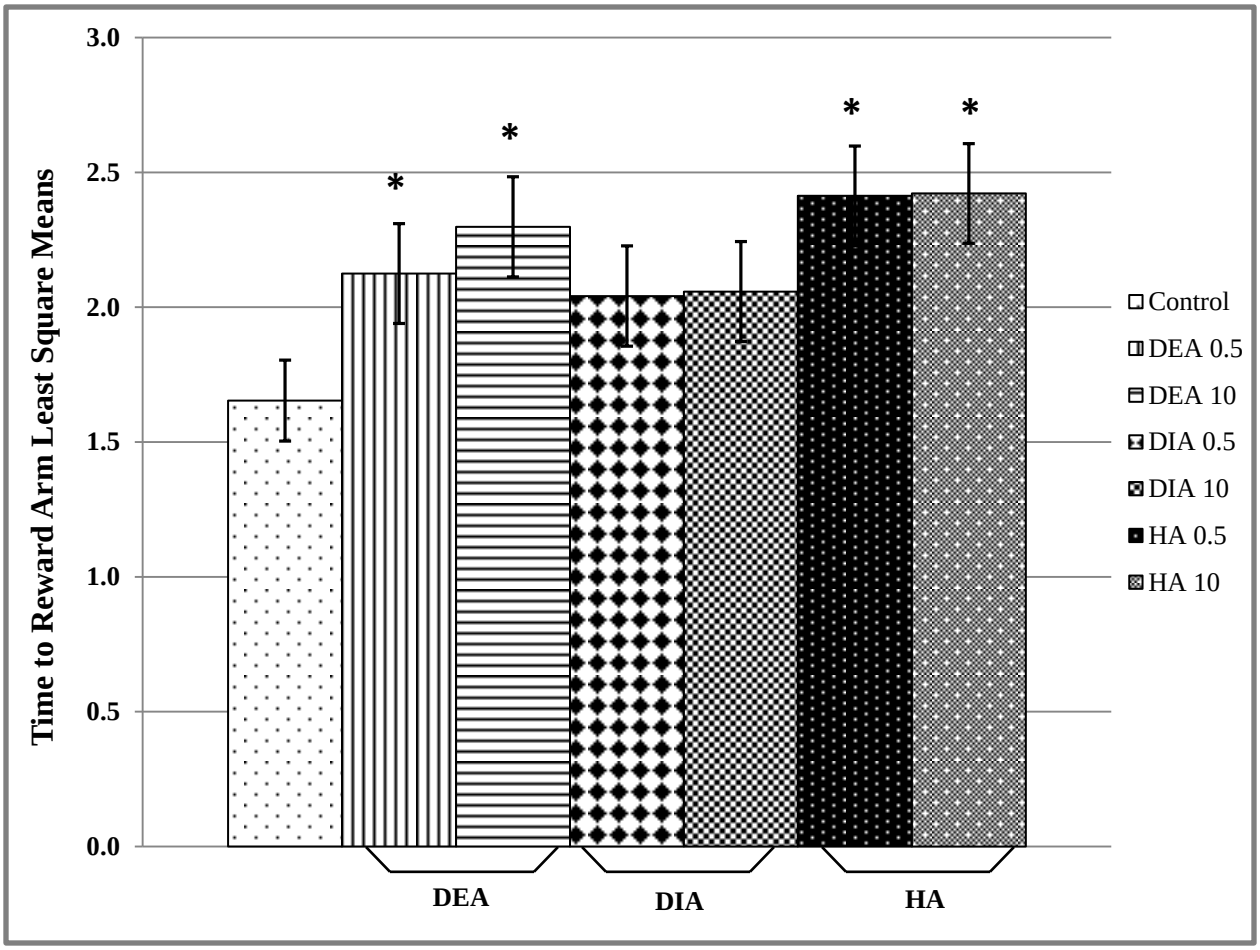

Figure 4.2. Time to reward arm LSMeans. * Indicate significance from control (n = 15-17), and error bars represent 95% confidence intervals.

- DEA 0.5 (n = 6-11): p = 0.0485
- DEA 10 (n = 7-11): p = 0.0.007
- DIA 0.5 (n = 5-11): p = 0.1053
- DIA 10 (n = 8-11): p = 0.0905
- HA 0.5 (n = 6-11): p = 0.0015
- HA 10 (n = 9-11): p = 0.0013

DIA treatments were not significantly different from control, and none of the degradate treatments were significantly different from each other, which may indicate that DIA is slightly less toxic than the other degradates (although not significantly so). DEA and HA treatment groups had significantly greater latency to reward arm than did control.

Table 4.3. Effect of Trial On Time to Reward for each degradate treatment group. * Indicates significant effect of trial (day). DEA 10, DIA 0.5, and HA 0.5 groups had a significant effect of trial (day) on latency to reward.

Treatment	**Fit: Chi-square / DF**	**p-value**
Control (n = 15-17)	1.07	0.0610
DEA 0.5 (n = 6-11)	0.97	0.5958
DEA 10 (n = 7-11)	1.05	0.0134 *
DIA 0.5 (n = 5-11)	1.03	0.0170 *
DIA 10 (n = 8-11)	1.16	0.5915
HA 0.5 (n = 6-11)	0.96	0.0022 *
HA 10 (n = 9-11)	0.96	0.7610

Table 4.4. Effect of Trial On Time to Reward Arm. * Indicates significant effect of trial (day). DIA 0.5 and HA 0.5 groups had a significant effect of trial (day) on time needed to locate reward arm.

Treatment	**Fit: Chi-square / DF**	**p-value**
Control (n = 15-17)	1.14	0.1014
DEA 0.5 (n = 6-11)	1.02	0.6587
DEA 10 (n = 7-11)	0.93	0.4077
DIA 0.5 (n = 5-11)	1.04	0.0139 *
DIA 10 (n = 8-11)	1.18	0.8965
HA 0.5 (n = 6-11)	0.96	0.0087 *
HA 10 (n = 9-11)	1.13	0.6899

Trial (day) had a significant effect on time to reward for crayfish exposed to DEA 10 µg/L, DIA 0.5 µg/L and HA 0.5 µg/L, and there was also a significant effect of trial on time to reward arm for two of these groups, DIA 0.5 µg/L and HA 0.5 µg/L. Interestingly, mean time to reward peaked around trial day 8 for DEA 10 µg/L treatment group (Figure 4.3). This may indicate that there is a time frame following exposure to DEA 10 µg/L during which toxic risks are most severe, as trial (day) was significant for this group. However, it is important to realize that regardless of trial day, DEA 10 µg/L treatment group continued to take longer to locate reward than did control, with a mean overall time to reward of 14 min. 35 sec. compared to 6 min. 56 sec. for control.

There was a significant effect of trial for time to reward, as well as for time to reward arm for animals exposed to DIA 0.5 µg/L. Upon viewing Figures 4.4 and 4.5, once can see that time for both variables tended to increase over trials/days for DIA 0.5 treatment group. Perhaps, if animals were exposed to DIA 0.5 µg/L for a longer period of time and if trials were continued, this trend would persist. It seems reasonable to assume so, as trial did have a significant effect. Therefore, perhaps animals exposed to DIA 0.5 µg/L would have differed significantly from control crayfish for time to reward and time to reward arm if trials were continued for a longer period of time. This further highlights the importance of doing repeated trial studies so that one can better elucidate the effects of length of exposure time of potential toxins.

There was also a significant effect of trial/day on times to reward and reward arm for animals exposed to HA 0.5 µg/L. Mean time to reward and mean time to reward arm, Figures 4.6 and 4.7 (respectively) increased with prolonged exposure. Thus, the longer animals were exposed to 0.5 µg/L of HA the more pronounced the toxin-induced deficits, which were significantly different from control.

Based on results presented in section 4.1, crayfish exposed to 0.5 µg/L and 10 µg/L of DEA or HA demonstrated significantly impaired abilities to locate food reward and reward arm. Also, as exposure time to HA 0.5 µg/L increased observed deficits became more pronounced, and there was a peak in toxic effects observed on Day 8 for DEA 10 treatment group. Furthermore, there is reason to suspect that if crayfish were exposed to DIA 0.5 µg/L for a longer period of time observed deficits would be significant from control.

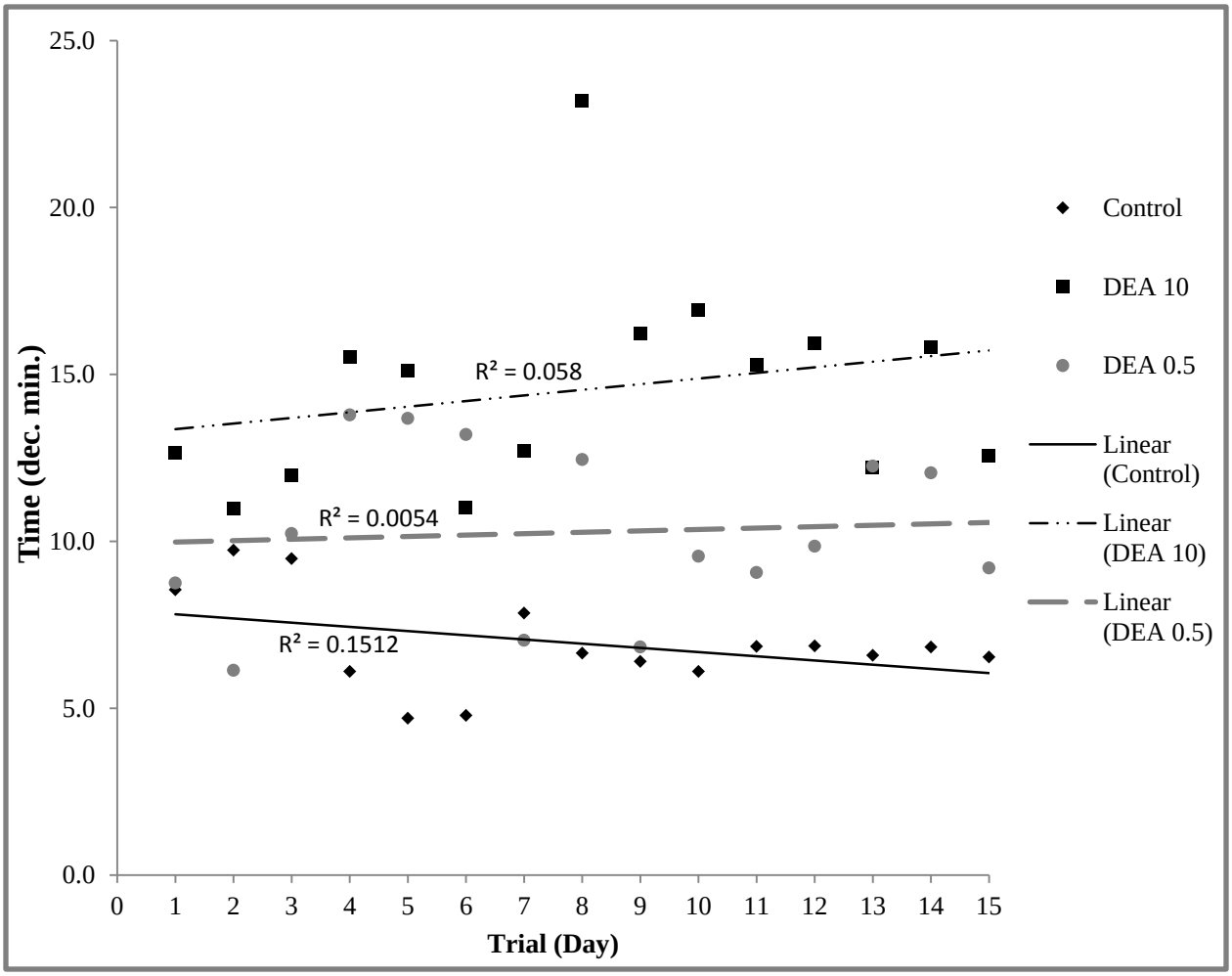

Fig. 4.3. Mean time to reward over repeated trials for control and DEA treated crayfish. Control: n = 15-17, DEA 0.5: n = 6-11, and DEA 10: n = 7-11. There was a significant effect of trial for DEA 10 µg/L treated crayfish. Latency to reward peaked on Day 8 for DEA 10 group, suggesting a possible time frame during which exposure to 10 µg/L of DEA generates the most toxic effects. Trendlines represent linear fit of data. DEA 0.5 & DEA 10 were both found to require significantly longer to locate reward compared to control (Table and Fig. 4.1).

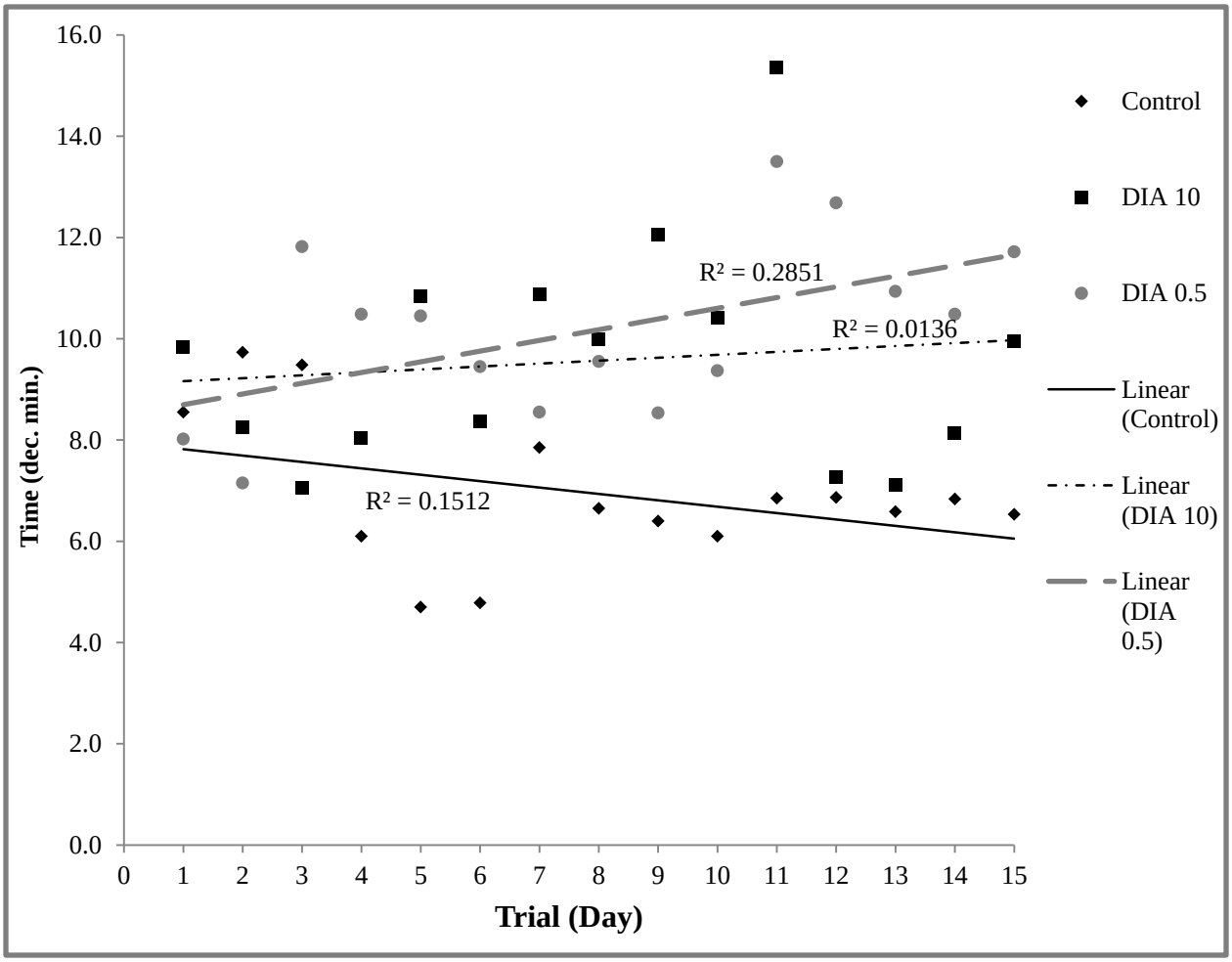

Fig. 4.4. Mean time to reward over repeated trials for control and DIA treatment groups. Control: n = 15-17, DIA 0.5: n = 5-11, and DIA 10: n = 8-11. Neither treatment group was significant from control, but there was a significant effect of trial on time to reward for DIA 0.5 µg/L treated crayfish; latency to reward increased, in general as trial/day progressed. Thus, it is possible that if trials had been continued the difference between control and DIA 0.5 in latency to reward may have proven to be significant. Trendlines represent linear fits of data.

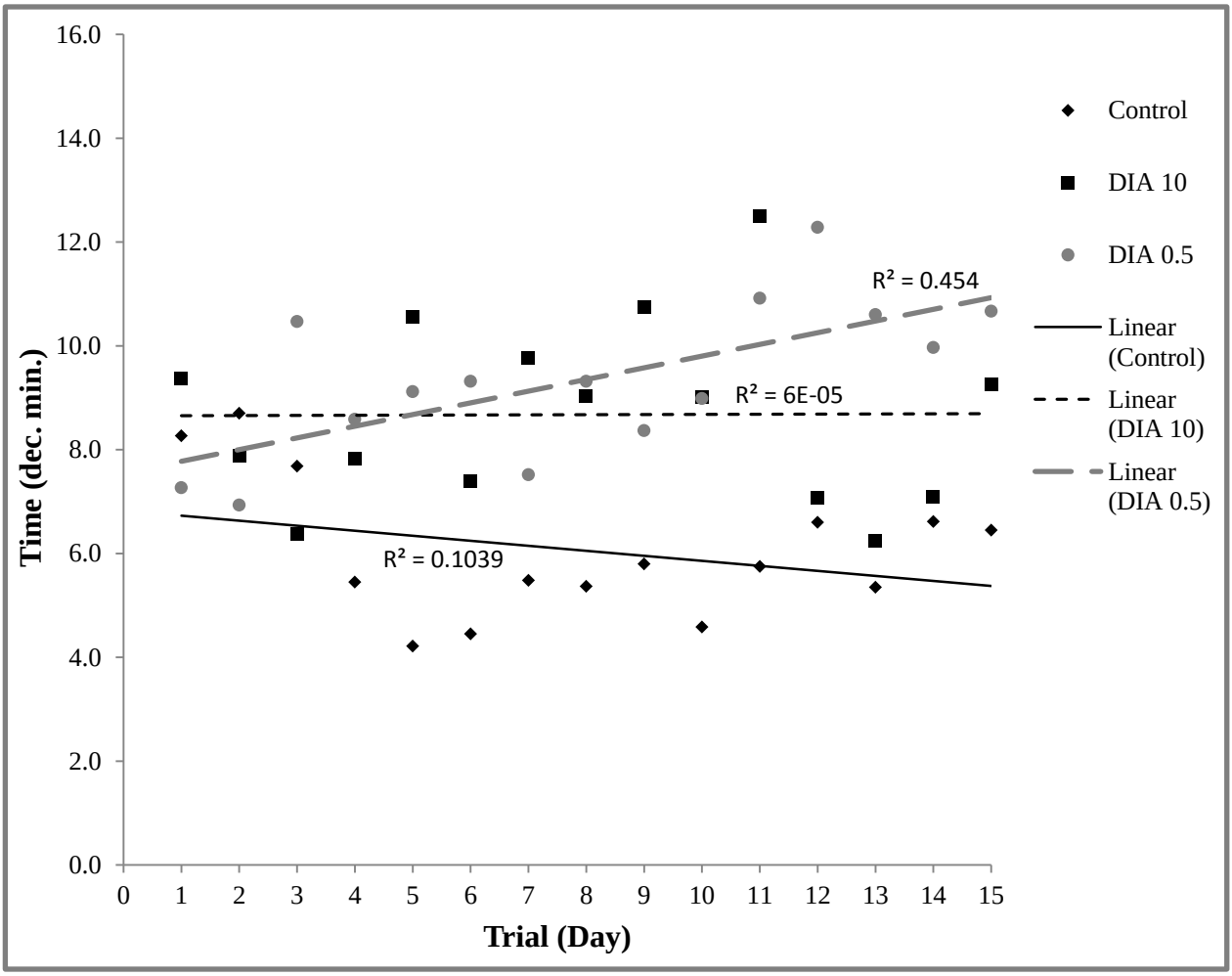

Fig. 4.5. Mean time to reward arm over repeated trials for control and DIA treatment groups. Control: n = 15-17, DIA 0.5: n = 5-11, and DIA 10: n = 8-11. Neither treatment group was significant from control, but there was a significant effect of trial on time to reward arm for DIA 0.5 µg/L treated crayfish. In general as trial/day progressed DIA 0.5 group took longer to locate reward arm. Thus, it is possible that if trials had been continued the difference between control and DIA 0.5 in latency to reward arm may have proven to be significant. Trendlines represent linear fits of data.

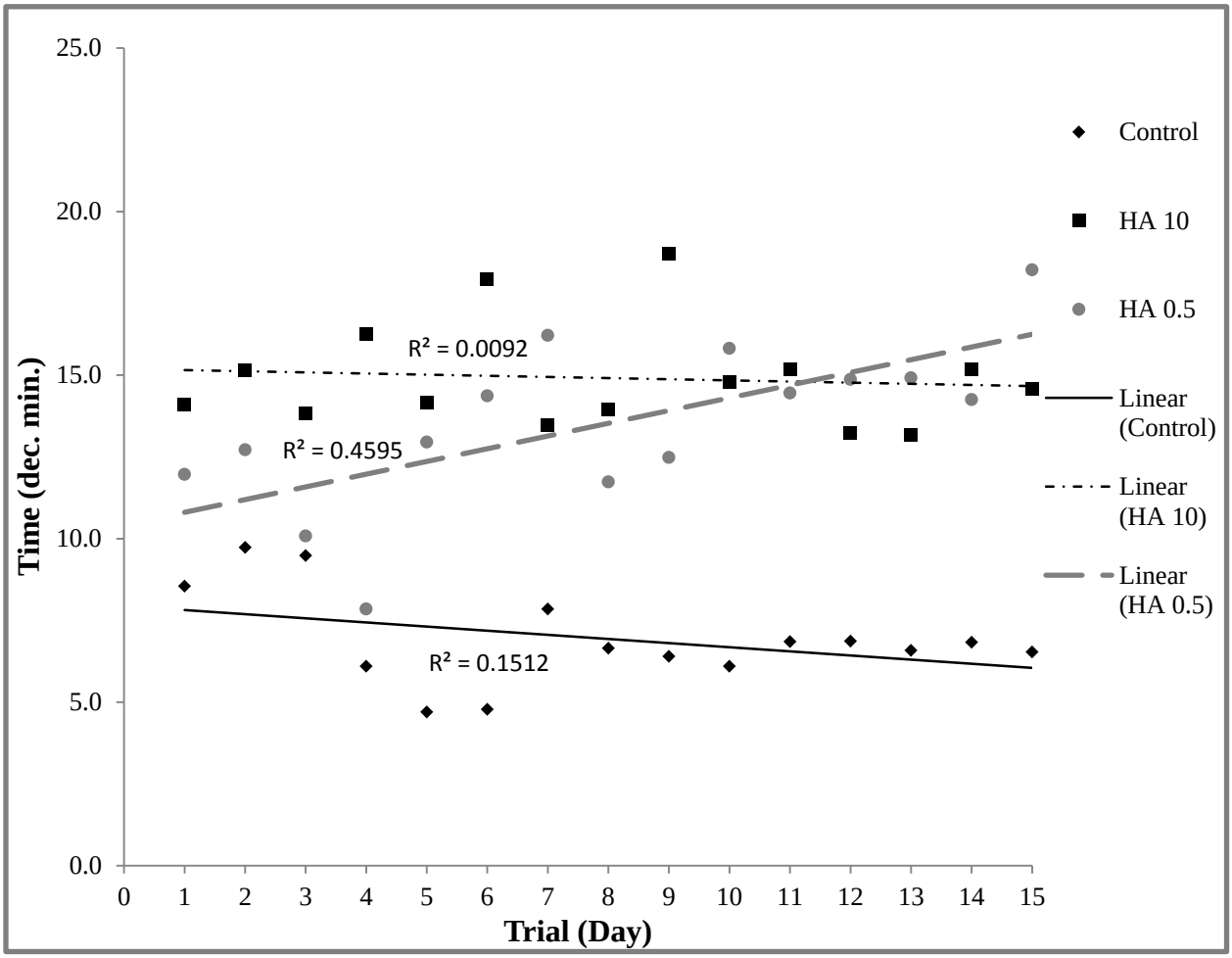

Fig. 4.6. Mean time to reward over repeated trials for control and HA treatment groups, which were both significantly different from control. Control: n = 15-17, HA 0.5: n = 6-11, and HA 10: n 9-11. There was a significant effect of trial for time to reward for HA 0.5 µg/L treated crayfish, which took longer to locate reward as trial progressed, suggesting that toxic effects of HA 0.5 µg/L increase with time. Trendlines represent linear fits of data.

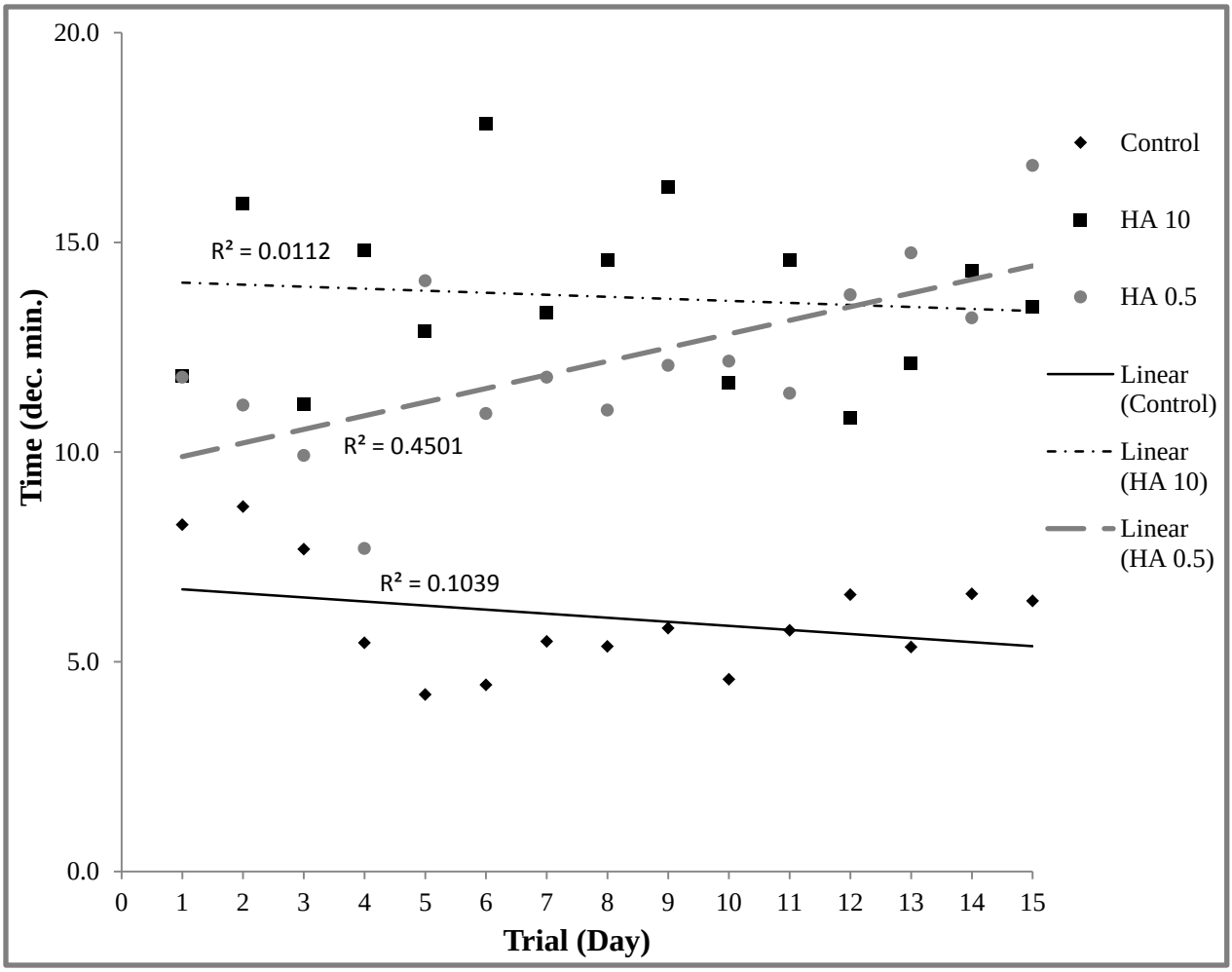

Figure 4.7. Mean Time to reward arm over repeated trials for control and HA treatment groups, which were both significantly different from control. Control: n = 15-17, HA 0.5: n = 6-11, and HA 10: n 9-11. There was a significant effect of trial for time to reward arm for HA 0.5 µ/L treated crayfish. HA 0.5 group took longer, in general to locate reward arm as trials progressed, which suggests that toxic effects of HA (at least at 0.5 µg/L) increase with time. Trendlines represent linear fits of data.

4.3. Percent of Crayfish to Not Locate Reward Arm

The model fit of mean percent of crayfish to not locate reward arm data was 0.73, and $p < .0001$. In order to determine the individual effects of atrazine degradate treatments on percent of crayfish to locate reward arm solutions for fixed effects and LSMeans tests were once again performed.

Degradate treatment groups' results for percent of crayfish to not locate reward arm offered quite a bit of variation between groups. Table 4.5 lists regression coefficients (estimates), standard error, p-values, alpha, and upper and lower 95 % confidence intervals for modeled percent of crayfish to not locate the reward arm data. All degradate treatment groups' regression coefficients were positive, excluding DIA 10 µg/L, thus indicating that most treatment groups had a greater overall percentage of crayfish that did not find the reward arm compared to control. However, half of the degradate treatment groups had significantly more animals not locate the reward arm compared to control ($p < 0.05$).

Degradate treatment groups that had a significantly greater percent of crayfish to not locate the reward arm included those exposed to DEA 10 µg/L, HA 0.5 µg/L and HA 10 µg/L. Similar to results for time to reward and time to reward arm data, neither DIA treatment group differed significantly from control. An additional group, DEA 0.5 µg/L, was not significantly different from control for this analysis, as well. A single treatment level of DEA was found to differ significantly from control, and both treatment levels of HA also had significant results. Results for this behavioral endpoint suggest that HA is the most toxic of the atrazine degradation products tested, as HA was the only degradate chemical for which results of both concentrations tested were significantly greater than control.

Results of LSMeans agree with degradate regression coefficients (Table 4.5); DEA 10 µg/L, HA 0.5 µg/L and HA 10 µg/L treatment groups' LSMeans were significantly greater than control's. Figure 4.9 and 4.10 include visual representations of significant LSMeans differences between degradate treatment groups. HA 10 µg/L and HA 0.5 µg/L LSMeans were both significantly greater than DEA 0.5 µg/L and DIA treatment groups (Figure 4.9). This may indicate that HA is the most toxic of the atrazine degradation products. Also, DEA 10 µg/L was significantly greater than DEA 0.5 µg/L, which was not significant from control, an indication that DEA may be much more toxic at the greater concentration. Furthermore, DEA 10 µg/L was also more toxic than both concentrations of DIA (Figure 4.10), which suggests that DIA is the least toxic of the degradates. Analysis of this behavioral endpoint, in particular, resulted in the greatest variation of results among treatment groups. Therefore, analysis of percent of crayfish to not locate reward arm may be the most beneficial behavioral endpoint for predicting the order of subthreshold toxicity. Results of degradate treatments' percent of crayfish to not locate reward arm LSMeans suggest a possible order of atrazine degradate toxicity as follows: HA ≥ DEA > DIA.

It is also noteworthy that ATR 0.5 and 200 µg/L treatment groups had significantly more animals not locate reward arm than did several degradate treatment groups, including DEA 0.5, DIA 0.5, DIA 10 µg/L (Figure 4.11). However, ATR 0.5 and 200 µg/L were not significantly different from either HA treatment group, while HA 10 was significantly greater than ATR 3. Thus, depending on the concentration being tested atrazine and HA are either comparably as toxic or HA is significantly more toxic (based on percent of crayfish to not locate reward arm data). Furthermore, for the same response variable, ATR 10 was significantly greater than DEA 0.5 and DIA 10. ATR 3, which was not significant from control, was also significantly less than

DEA 10 and significantly greater than DIA 10 (Figure 4.11). Therefore, as mentioned previously, test matters. Analysis of percent of crayfish to not locate reward arm data generated, by far, the most diverse results, but this analysis may be useful in predicting order of toxicity. As explained previously, there is evidence that HA ≥ DEA > DIA, and depending on concentration tested ATR ≤ HA. However, if one only considers comparisons between equal concentrations, i.e. at 0.5 or 10 µg/L, atrazine was found to be comparably as toxic as HA at both concentration levels and was also comparable to DEA 10. Based on this criteria, ATR ~ HA in toxicity. Thus, the suggested order of toxicity is ATR ~ HA ≥ DEA > DIA.

Table 4.5. Percent Crayfish to Not Locate Reward Arm Solutions for Fixed Effects. * indicates significance from control. Half of the degradate treatment groups had significantly more crayfish not locate reward arm compared to control. Alpha = 0.05.

Treatment	Estimate	Standard Error	p	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
DEA 0.5 (n = 6-11)	0.1173	0.4118	0.7762	-0.6962	0.9307
DEA 10 (n = 7-11)	1.6620	0.3969	<.0001 *	0.8779	2.4461
DIA 0.5 (n = 5-11)	0.5778	0.4049	0.1556	-0.2220	1.3776
DIA 10 (n = 8-11)	-0.2039	0.4187	0.6270	-1.0311	0.6233
HA 0.5 (n = 6-11)	1.3991	0.3982	0.0006 *	0.6125	2.1856
HA 10 (n = 9-11)	1.7083	0.3967	<.0001 *	0.9246	2.4921

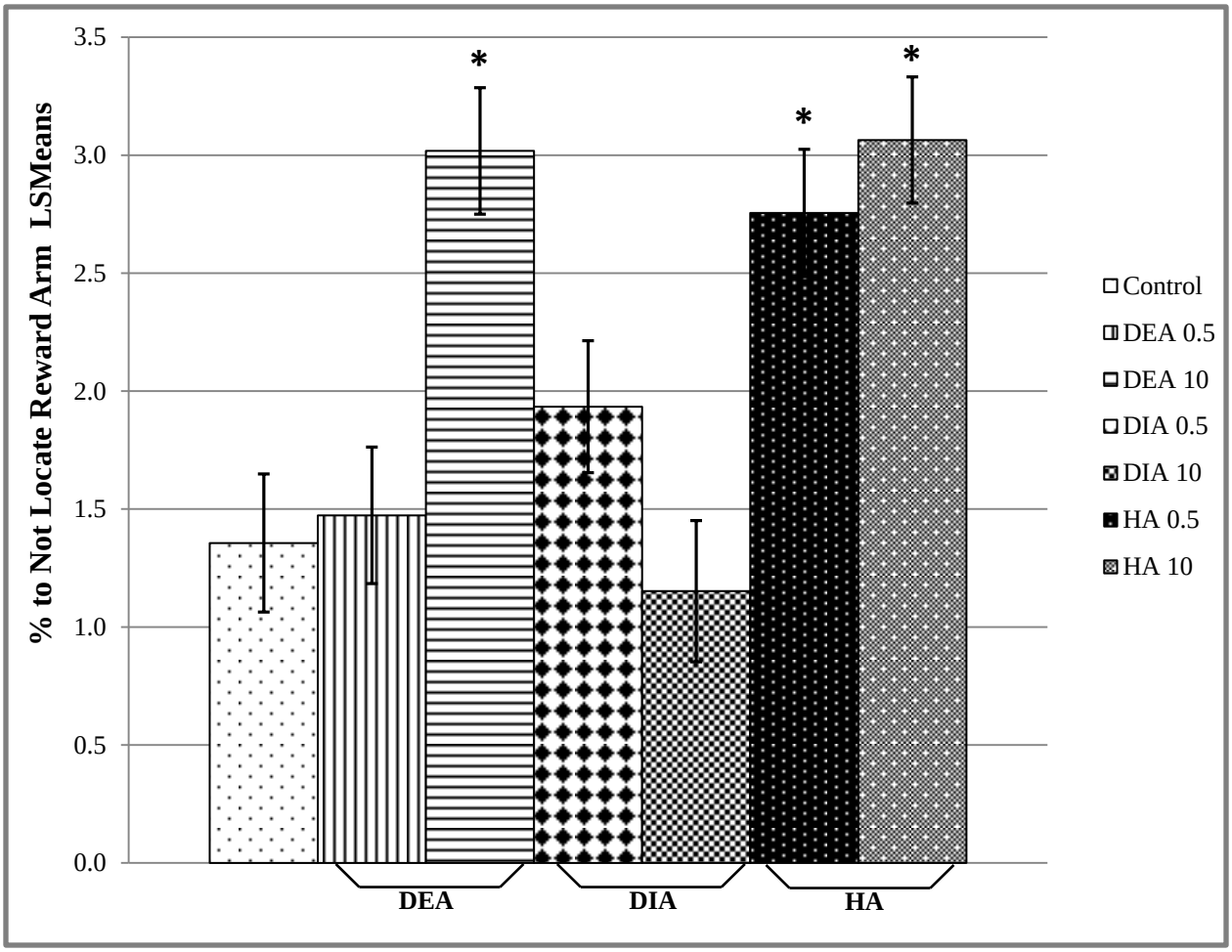

Fig. 4.8. Percent of crayfish to not locate reward arm LSMeans for control and all degradate treatment groups. . * Indicate significance from control (n = 15-17), and error bars represent 95% confidence intervals.

- DEA 0.5 (n = 6-11): p = 0.7762
- DEA 10 (n = 7-11): p < .0001
- DIA 0.5 (n = 5-11): p = 0.1556
- DIA 10 (n = 8-11): p = 0.627
- HA 0.5 (n = 6-11): p = 0.0006
- HA 10 (n = 9-11): p < .0001

Both HA treatment groups and DEA 10 had significantly more crayfish not locate reward arm compared to control crayfish.

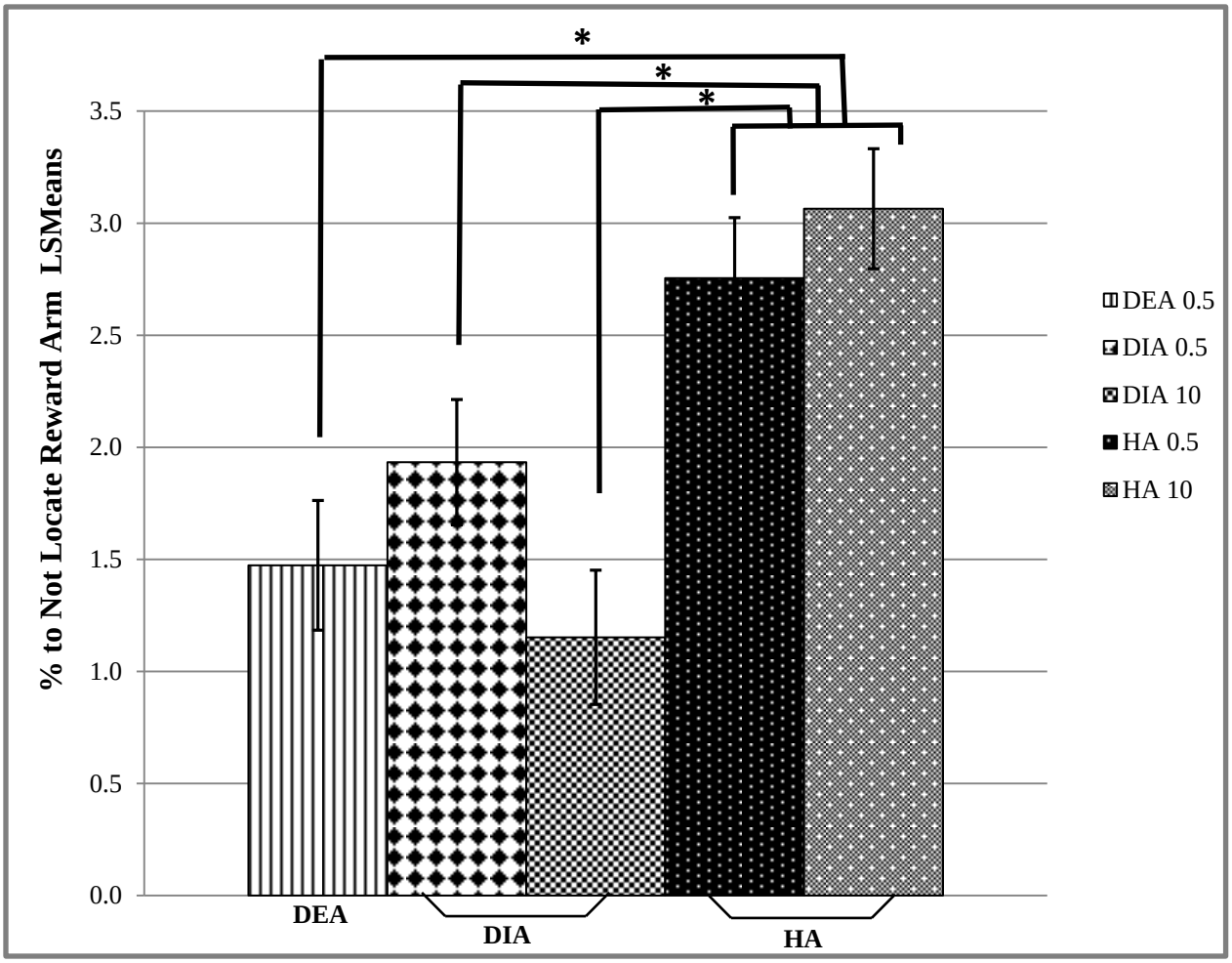

Fig. 4.9. Percent of crayfish to not locate reward arm LSMeans. Graph shows treatments that were significantly different from HA treatment groups, indicated by *. Error bars represent 95% confidence intervals

Pairwise comparisons: to HA 0.5 (n = 6-11) and to HA 10 (n = 9-11):

- DEA 0.5 (n = 6-11): p = 0.0015 and < .0001
- DIA 0.5 (n = 5-11): p = 0.0362 and = 0.004
- DIA 10 (n = 8-11): p = 0.0001 and < .0001

Results suggest that HA is most toxic of degradation products.

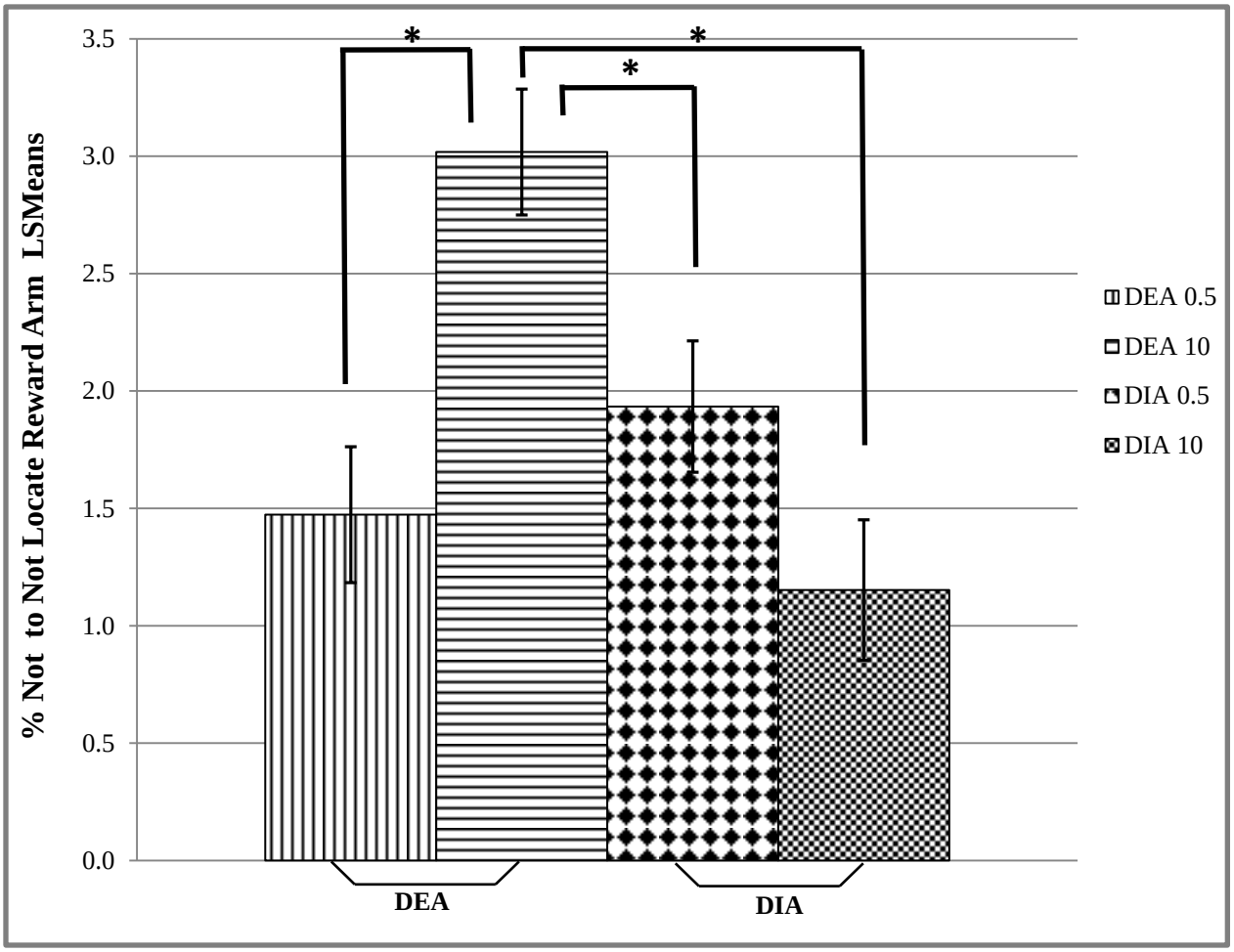

Fig. 4.10. Percent of crayfish to not locate reward arm LSMeans. Graphs shows treatments that were significantly different from DEA 10 µg/L, indicated by *. Error bars represent 95% confidence intervals.

Pairwise comparisons to DEA 10 (n = 7-11):

- DEA 0.5 (n = 6-11) : p = 0.0001
- DIA 0.5 (n = 5-11): p = 0.0058
- DIA 10 (n = 8-11): p < .0001

Results indicate that DEA is significantly more toxic at the greater concentration; also, DEA is more toxic than DIA. In conjunction with Fig. 4.9, this provides evidence that the order of degradate toxicity is HA > DEA > DIA.

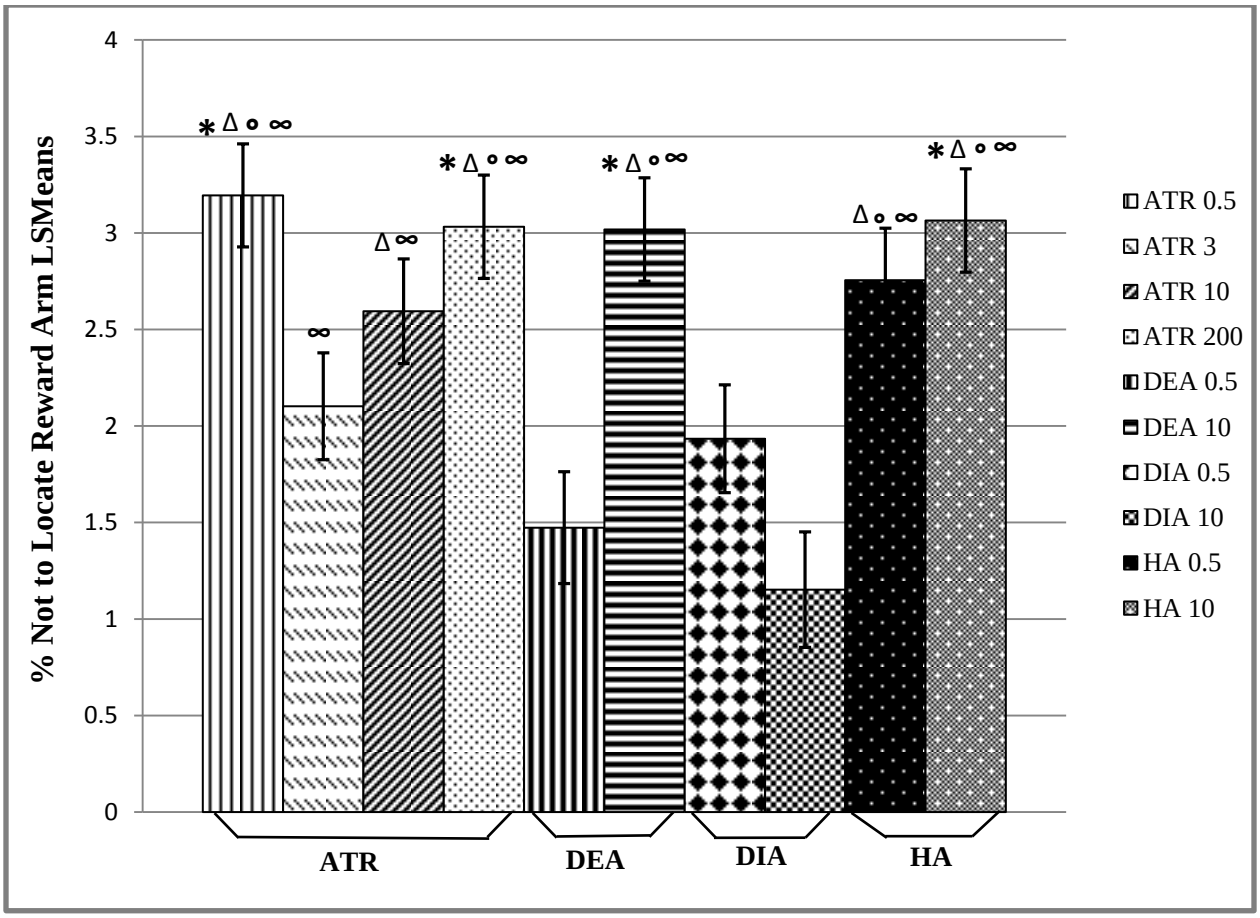

Fig. 4.11. Percent of crayfish not to locate reward arm LSMeans comparing atrazine and degradate treatment groups in pairwise fashion. P-values are listed in Table A.3. Error bars represent 95 % confidence intervals. Sample sizes were as follows: ATR 0.5: n = 10-11, ATR 3: n = 10-12, ATR 10: n = 7-10, ATR 200: n = 8-12, DEA 0.5: n = 6-11, DEA 10: n = 7-11, DIA 0.5: n = 5-11, DIA 10: n = 8-11, HA 0.5: n = 6-11, HA 10: n = 9-11.

- Represents treatment was significantly > ATR 3 : *****
- Represents treatment was significantly > DEA 0.5: Δ
- Represents treatment was significantly > DIA 0.5: °
- Represents treatment was significantly > DIA 10: ∞

There were a lot of significant differences between treatment groups. Both DIA treatment groups and DEA 0.5 were found to generate the least deficits in this response variable, followed by ATR 3. DEA was significantly more toxic at the higher concentration. ATR ≤ HA groups & DEA 10, depending on concentration tested. This analysis may be the most useful

4.4. Time Spent in Under Cover Area of the T-Maze

The model fit of under cover data was 1.04, and p was < .0001, indicating a very significant effect of treatment on time spent in under cover area. In order to determine the individual effects of degradate treatments on time spent under cover solutions for fixed effects and LSMeans tests were performed.

Each degradate treatment was found to have a positive regression coefficient, as well as a significant p-value (Table 4.6). Thus, all groups of crayfish exposed to a degradate treatment spent significantly more time in the under cover area of the maze than did control animals.

Results of degradate treatment groups' modeled under cover data LSMeans (Figure 4.12) agree with results for solutions of fixed effects. Note that none of the degradate treatments were found to be significantly different from each other, which differs from previous analyses. Overall, the analysis of degradate treatment groups' time spent under cover data suggests that each degradate treatment group did spend significantly more time in this area at the base of the maze, which indicates that crayfish exposed to 0.5 and 10 µg/L of DEA, DIA and HA were less bold and explorative than untreated animals and preferred the covered area of the maze, perhaps as a means of avoiding potential predators or due to increased lethargy.

Compared to atrazine treatment groups, both HA treatment groups spent significantly more time under cover than did animals exposed to atrazine 200 µg/L, providing further evidence that HA is more toxic than atrazine at certain concentrations. ATR 200 also spent significantly less time under cover than DEA 10; however, ATR 0.5 was significantly greater than DEA 0.5 (refer to tables and figures in Appendix). So, once again, the order of toxicity depends on the concentrations being tested, as well as on the behavioral endpoint being assessed.

Table 4.6. Under Cover Solutions for Fixed Effects of Degradate Treatment Groups. Every degradate treatment groups spent significantly longer (based on regression coefficient estimates and their respective p-values) under cover than did control. * Indicates significance from control. Alpha = 0.05.

Treatment	Estimate	Standard Error	p	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
DEA 0.5 (n = 6-11)	1.0388	0.3989	0.0093 *	0.2563	1.8212
DEA 10 (n = 7-11)	1.5681	0.4000	<.0001 *	0.7836	2.3526
DIA 0.5 (n = 5-11)	1.1951	0.4002	0.0029 *	0.4103	1.9800
DIA 10 (n = 8-11)	1.1913	0.3987	0.0029 *	0.4092	1.9733
HA 0.5 (n = 6-11)	1.6694	0.3976	<.0001 *	0.8894	2.4493
HA 10 (n = 9-11)	1.6241	0.3986	<.0001 *	0.8423	2.4059

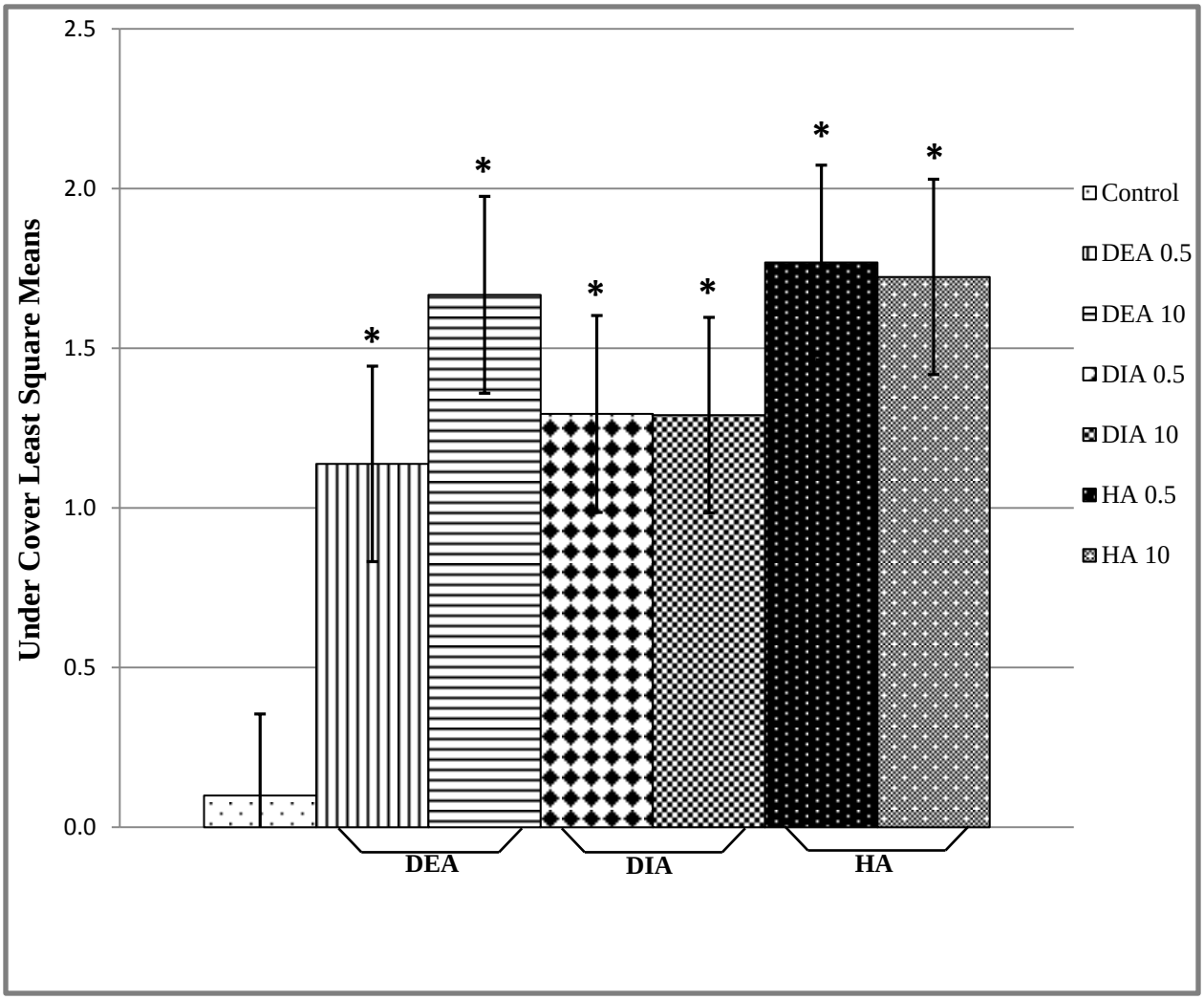

Fig. 4.12. LSMeans of under cover times for control and degradate treatment groups. * indicates a significant difference from control (n = 15-17). Error bars represent 95% confidence intervals.

- DEA 0.5 (n = 6-11): $p = 0.0093$
- DEA 10 (n = 7-11): $p < .0001$
- DIA 0.5 (n = 5-11): $p = 0.0029$
- DIA 10 (n = 8-11): $p = 0.0029$
- HA 0.5 (n = 6-11): $p < .0001$
- HA 10 (n = 9-11): $p < .0001$

All treatments differed significantly from control, but did not differ from each other.

In order to determine if length of time animals were exposed to various levels of DEA, DIA and HA may have had an effect on time spent under cover and/or if familiarity with the maze increased explorative behavior effect of trial on time spent under cover was tested for each degradate treatments group. Table 4.7 lists the model fit for each treatment, in which effect was trial and time under cover was response, as well each models' respective p-values (type III tests).

Trial, or day, was found to have a significant effect on the amount of time spent in the under cover area of the maze by DEA 10 µg/L, DIA 0.5 µg/L and HA 0.5 µg/L treated animals. Interestingly, there was also a significant effect of trial on time spent under cover by control crayfish.

Control crayfish, in general, spent less time in the under cover area as trail, or day, progressed at least until trial day 12 (Figures 4.13 – 4.15). Trial had a significant effect on time spent under cover for control crayfish. Thus, control animals spent significantly less time under cover as trial (day) progressed; this may indicate that as animals spent more time in the maze and became familiar with it they became more explorative and bold, up until a point. Perhaps the slight increase in under cover for the last few trials shows that crayfish simply became bored with the maze after a while. It's difficult, and risky, to try to explain all motives behind a group of animals' behavior as much variation naturally exists. Regardless of slight increases in time under cover near the end of the trial period, it is obvious that untreated crayfish, in general, become significantly bolder and more explorative as they became more familiar with the maze. Additionally, it is also possible to extend this interpretation to infer that perhaps significantly decreased time under cover area over repeated trials may be evidence of learning in control crayfish. Possibly, as animals became more familiar with their environment and learned that it

was not threatening (lacked potential predators) they became increasingly explorative and bold as a result.

There was also a significant effect of trial on time spent under cover by crayfish exposed to DEA 10 µg/L. Referring to Figure 4.14 this group of crayfish had a time-frame, trials 8 to 10, during which they spent the most time in the under cover area. This may suggest that there is a particular time frame following exposure to DEA 10 µg/L during which toxic effects are most severe. This observation was also made for DEA 10 µg/L treated crayfish for time to reward over repeated trials (Fig. 4.3), in which mean time to reward was greatest during trial 8. Thus, there is added support for a time-frame following exposure during which toxic effects of DEA 10 µg/L are most severe.

There was also a significant effect of trial, or day, on time spent under cover for crayfish exposed to DIA 0.5 µg/L and HA 0.5 µg/L. For both of these treatments, mean time under cover increased as trials progressed (Figures 4.14 & 4.15), with a peak amount of time spent under cover on trial day 12. Furthermore, these observations are supported by those made for the same treatments for the response variables time to reward and time to reward arm (Figs. 4.4 – 4.7), in which both treatment groups required longer to find reward and reward arm. These results in conjunction suggest that detrimental subthreshold effects of DIA 0.5 µg/L and HA 0.5 µg/L increase with time and may have a peak day during which toxicity is most pronounced, which once again stresses the importance of multiple analyses and longitudinal studies for comprehensive interpretation of environmental contaminants' toxicity.

Table 4.7. Effect of Trial on Time Spent in Under Cover Area for Control and Degradate Treatment Groups. There was a significant effect of trial/day on time under cover for control, DEA 10, DIA 0.5, and HA 0.5 groups.

Treatment	**Fit: Chi-square / DF**	**p-value**
Control (n = 15-17)	1.02	0.0004 *
DEA 0.5 (n = 6-11)	0.97	0.9567
DEA 10 (n = 7-11)	0.99	0.0049 *
DIA 0.5 (n = 5-11)	1.10	<.0001 *
DIA 10 (n = 8-11)	1.23	0.7389
HA 0.5 (n = 6-11)	0.87	0.0045 *
HA 10 (n = 9-11)	1.14	0.3379

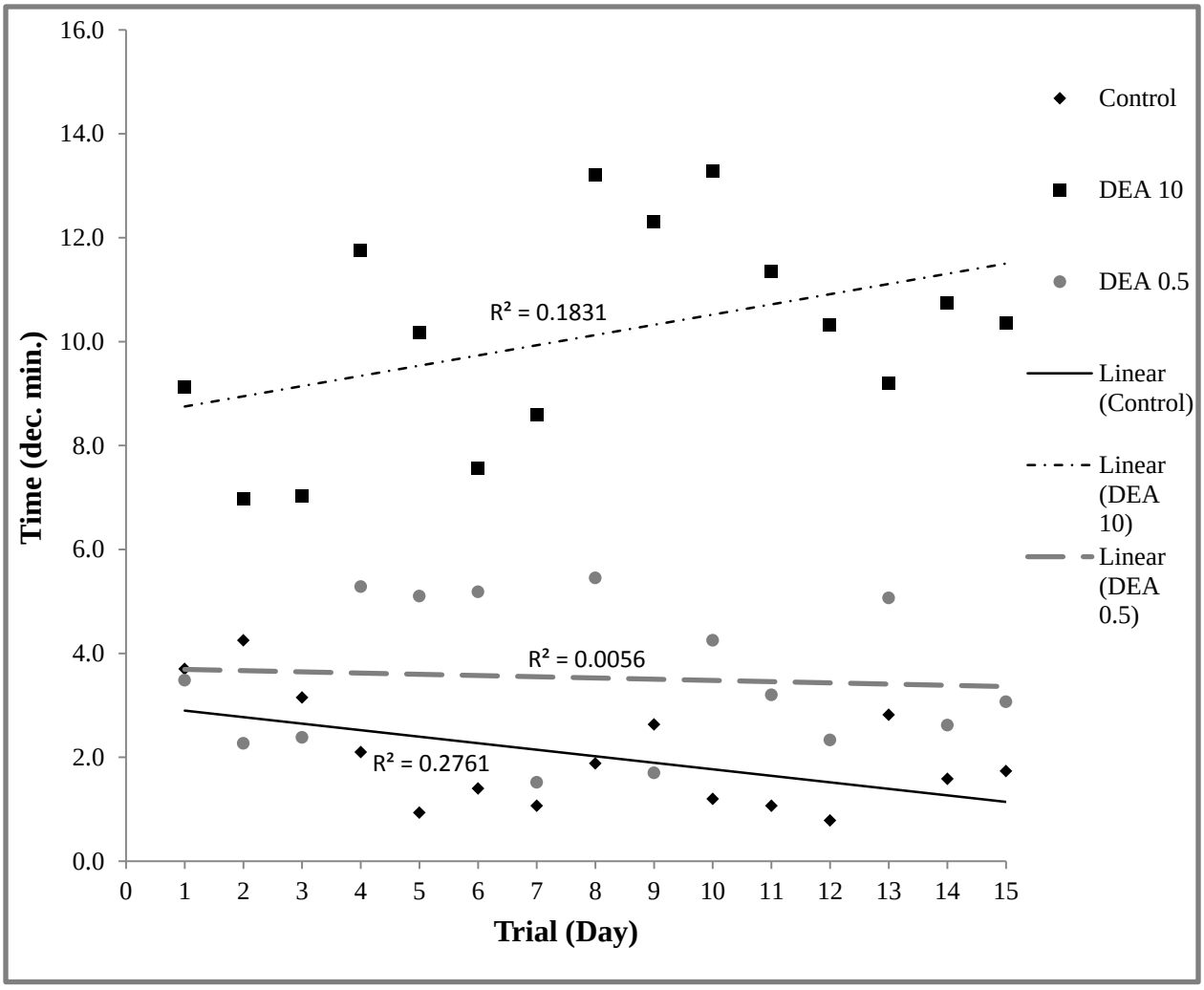

Fig. 4.13. Mean time under cover over entire trial period (15 days) for control and DEA treatment groups. Control: n = 15-17, DEA 0.5: n = 6-11, and DEA 10: n = 7-11. Trendlines represent linear fit of data. DEA 0.5 & 10 µg/L spent significantly longer under cover compared to control (Table 4.6 & Fig. 4.11). There was a significant effect of trial on time under cover for control and DEA 10 treated crayfish. Control crayfish spent significantly less time under cover, at least up until trial 12, indicating that as they became more familiar with their environment untreated crayfish became bolder and began to explore the maze more. DEA 10 group had a time frame during which time spent under cover was at a maximum, trials 8 to10, suggesting a period following exposure during which toxic effects of DEA 10 µg/L are most pronounced. Overall, treated crayfish exhibited impaired boldness and increased lethargy compared to control crayfish.

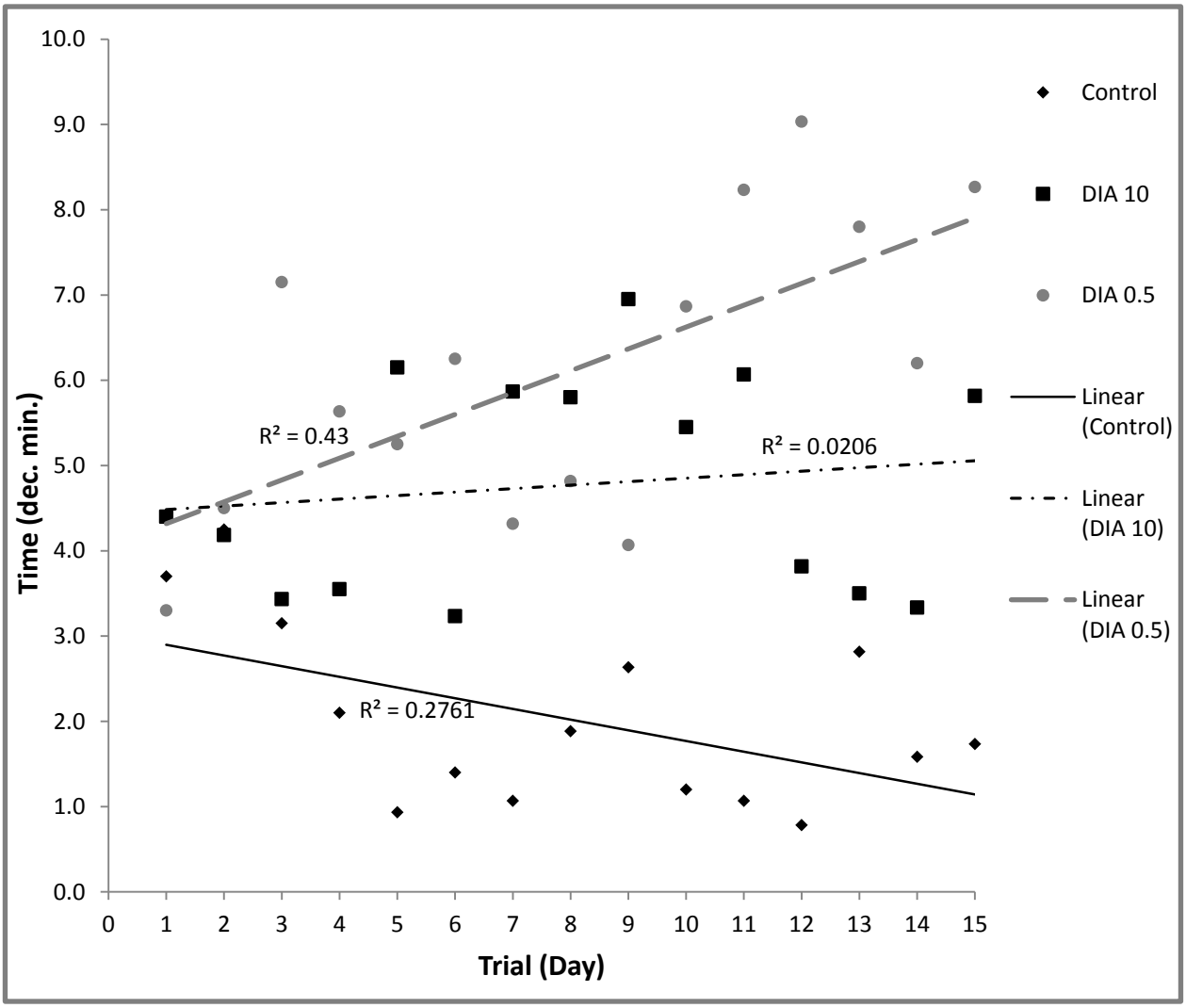

Fig. 4.14. Mean time under cover over entire trial period (15 days) for control and DIA treatments groups. Trendlines represent linear fits of data Control: n = 15-17, DIA 0.5: n = 5-11, and DIA 10: n = 8-11. DIA 0.5 & 10 µg/L spent significantly longer under cover compared to control (Table 4.6 & Fig. 4.11). There was a significant effect of trial on time under cover for control and DIA 0.5 treated crayfish, which in general spent longer under cover as trial progressed. Time under cover peaked on day 12 for DIA 0.5 group, suggesting a period following exposure during which toxic effects of DIA 0.5 µg/L are most pronounced. Control crayfish spent significantly less time under cover as trials progressed for most of the trial period (12 days). Overall, DIA treated crayfish exhibited impaired boldness and increased lethargy compared to control crayfish..

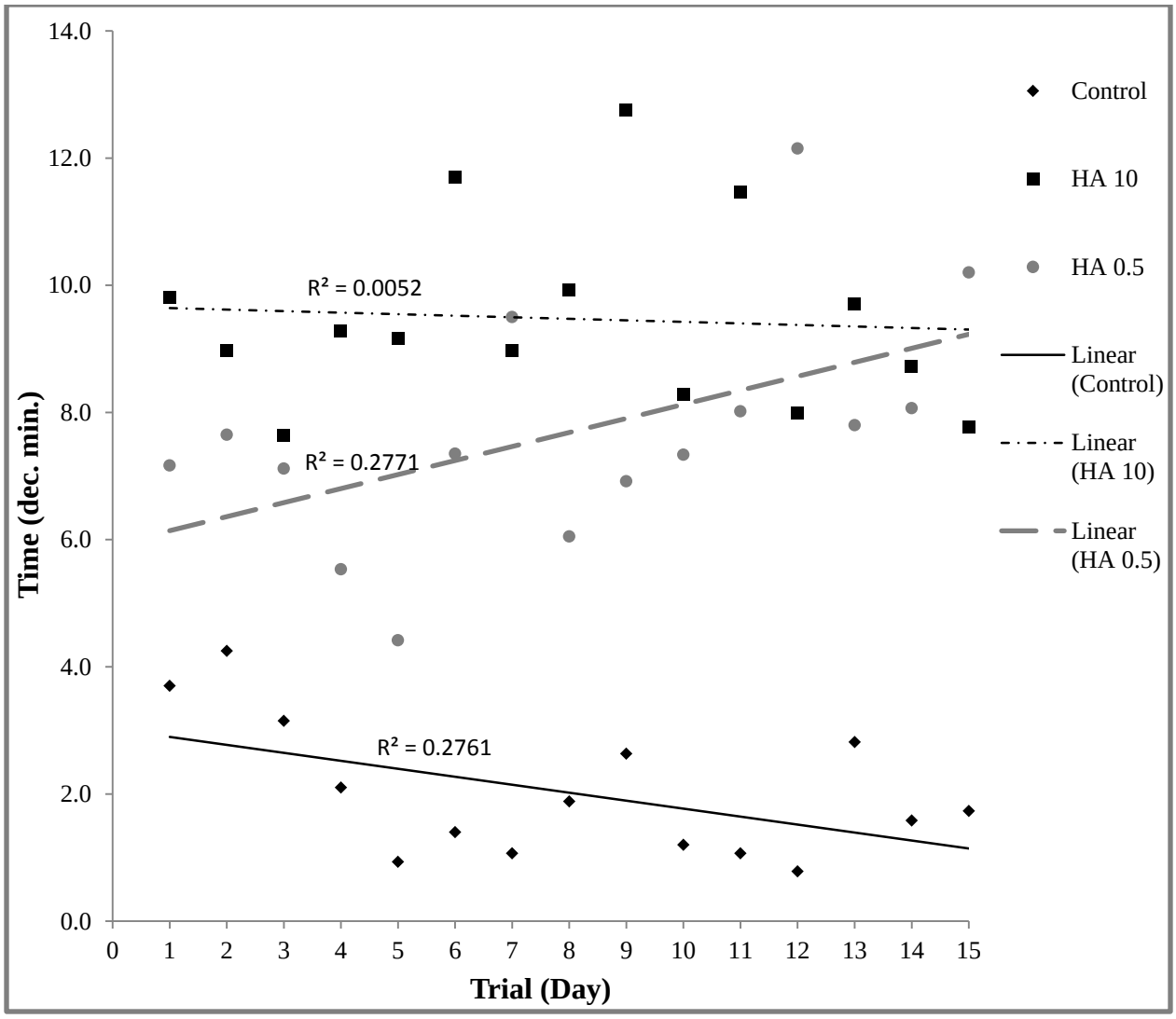

Fig. 4.15. Mean time under cover over entire trial period (15 days) for control and HA treated animals. Trendlines represent linear fits of data Control: n = 15-17, HA 0.5: n = 6-11, and HA 10: n = 9-11. HA treated crayfish spent significantly longer under cover compared to untreated crayfish (Table 4.6 & Fig. 4.11). There was a significant effect of trial on time under cover for control and HA 0.5 treated crayfish, which spent increasing amounts of time under cover as trial progressed, in general, with a peak time under cover on trial day 12. These results suggest that control crayfish spent significantly less time under cover (up to trial 12), while HA 0.5 group spent significantly more time under cover, as trials (days) progressed. Thus, untreated crayfish became bolder as they became more familiar with their environment, while HA treated crayfish did not. Furthermore, toxic effects of HA 0.5 became more pronounced with prolonged exposure.

4.4. Time Needed to Complete ~ 180° Turnarounds

The model fit for ~ 180° turnaround data was 0.89, and the p-value for treatment (Type III Tests) was < .0001. Treatment had a very significant effect on the time needed for crayfish to complete roughly 180° turnarounds. Compared to control every degradate treatment group of crayfish took longer, as indicated by positive regression coefficients (estimate values in Table 4.8), to complete turnarounds. Furthermore, these elevated values were significantly different from control, based on p-values < .0001 for each atrazine degradate treatment group.

Table 4.8. ~ 180° Turnaround Solutions for Fixed Effects. Every degradate treatment group took significantly longer than control to complete turnarounds, indicated by *. Alpha = 0.05.

Treatment	Estimate	Standard Error	p	Lower	Upper
Control (n = 15-17)	0	.	.	.	.
DEA 0.5 (n = 6-11)	0.6489	0.1029	<.0001 *	0.4469	0.8509
DEA 10 (n = 7-11)	0.6421	0.1052	<.0001 *	0.4357	0.8486
DIA 0.5 (n = 5-11)	0.5471	0.1085	<.0001 *	0.3342	0.7601
DIA 10 (n = 8-11)	0.6483	0.1014	<.0001 *	0.4492	0.8473
HA 0.5 (n = 6-11)	0.7095	0.1041	<.0001 *	0.5052	0.9138
HA 10 (n = 9-11)	0.5853	0.1074	<.0001 *	0.3745	0.7962

Results of LSMeans of modeled turnaround data are in concordance with the regression coefficients and their p-values. Based on LSMeans results, animals exposed to all degradate treatment levels required significantly longer to perform ~ 180° turnarounds. This suggests that as a result of exposure to DEA, DIA or HA at 0.5 or 10 µg/L crayfish had impaired motor control (Figure 4.16). These results are similar to those observed for atrazine treatment groups, in which all treatment levels were significant from control. Additionally, there was not a significant different between degradate treatment groups for turnaround times (Fig. 4.16).

Some atrazine treatment groups proved to be significantly different from several degradate treatments for time needed to complete ~ 180° turnarounds (refer to tables and figures in Appendix). Crayfish exposed to atrazine 200 µg/L took significantly less time to perform turnarounds than did the following treatment groups: HA 0.5, both DEA groups, and DIA 10. Additionally, atrazine 3 µg/L took significantly less time to perform turnarounds than did HA 0.5. Thus, perhaps atrazine at 0.5 and 10 µg/L is at least slightly, but not significantly, more toxic than atrazine at 3 or 200 µg/L, based on the fact that the two later atrazine concentrations were significantly less toxic (for turnaround time) than several degradate treatment groups. Also, recall that for some previous behavioral endpoints analyzed there was evidence that HA > DEA > DIA in toxicity, but not so for turnaround time. Therefore, once again, variation in results among different analyses highlights the importance of performing multiple behavioral analyses so that one may gain a more comprehensive and less biased understanding of toxic effects due to environmental contaminants at sublethal levels.

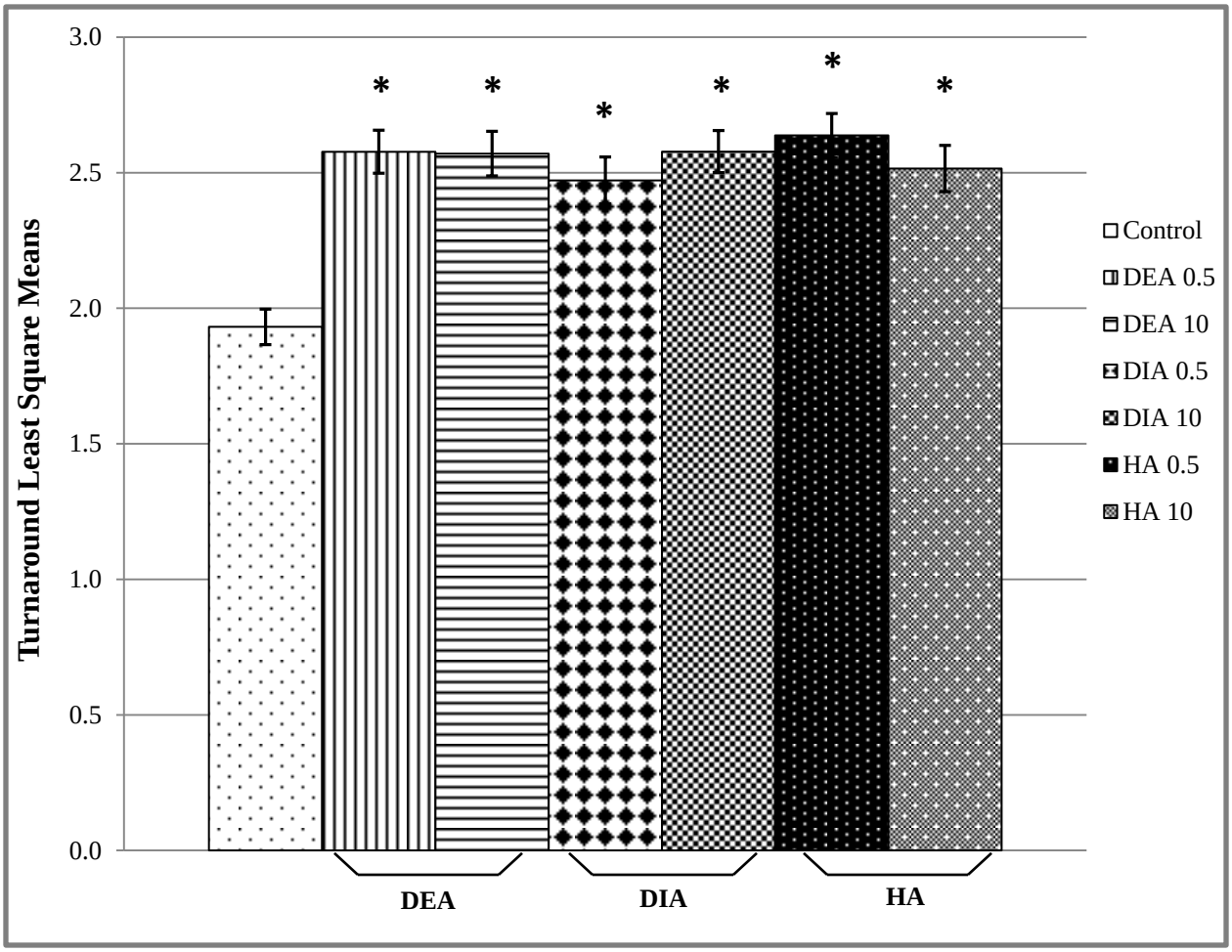

Fig. 4.16. LSMeans of ~180° turnaround times. Error bars represent 95% confidence intervals. P-value for each treatment compared to control was <.0001. Sample sizes: control: n = 15-17, DEA 0.5: n = 6-11, DEA 10: n = 7-11, DIA 0.5: n = 5-11, DIA 10: n = 8-11, HA 0.5: n = 6-11, and HA 10: n = 9-11.

All degradate treatment groups took significantly longer than control (indicated by *) to perform turnarounds, which may indicate impaired motor control due to exposure to atrazine's primary environmental degradation products.

Chapter 5. Conclusions and Implications

Several behavioral endpoints were tested, including time to reward, time to reward arm, percent of animals to not locate reward arm, time spent under cover, and time needed to complete ~180° turnarounds. There was quite a bit of variation in results among atrazine, DEA, DIA and HA treatment groups between many of these analyses. Thus, it is risky to make judgments on toxicity based on a single test. Multiple analyses offer a comprehensive understanding of the overall toxic effects of contaminants on organisms and the ecosystem. Although there was some variation in results between analyses, each treatment level of atrazine, DEA, DIA and HA resulted in significant deficits compared to untreated crayfish for at least two behavioral analyses, providing evidence that each of these contaminants induce adverse effects at low, environmentally realistic doses in *P. clarkii* and are therefore toxic. Furthermore, evidence that each of atrazine's degradates are toxic suggests that the risks associated with atrazine's presences in the environment do not merely subside as the herbicide is degraded.

Additionally, it is beneficial to do repeated trial studies so that one may gain a better understanding of contaminants' toxic effects over time. In fact, there was a significant effect of trial/day for some of the treatment groups for certain behavioral analyses. DIA 0.5 µg/L treated crayfish did not take significantly longer than control animals to locate the reward or reward arm. However, there was a significant effect of trial for both time to reward and time to reward arm for DIA 0.5 µg/L treated crayfish. This group of animals took significantly longer over repeated trials, in general, to locate the reward and reward arm. Thus, it is plausible that if the experiment had been continued for a few more days DIA 0.5 µg/L treated crayfish would have continued to progressively take longer to locate reward and reward arm, and perhaps, eventually the

difference between control and this treatment group for times to reward and reward arm would have been significant.

There was also evidence that toxicant-induced behavioral deficits increased with prolonged exposure for various other treatment groups. Atrazine 3 µg/L, DEA 10 µg/L, and HA 0.5 µg/L treated crayfish took significantly longer as trial progressed to locate reward and/or reward arm. Another treatment group, ATR 0.5 µg/L also exhibited a significant effect of trial on time to reward; however, interpretation of any trend from that data set was impossible as the data were incredibly chaotic. Control crayfish spent significantly less time under cover (up to trial 12) as trial progressed, which is evidence that as untreated animals became more familiar with the maze they became significantly more explorative and bold as trial, or day, progressed. A significant effect of trial on time spent under cover was observed for crayfish exposed to DEA 10 µg/L, DIA 0.5 µg/L, and HA 0.5 µg/L; thus, these treatment groups became significantly less explorative and bold as the amount of time animals were exposed to these contaminants increased. There was also evidence of time frames following exposure during which toxic effects were most pronounced. DEA 10 µg/L, DIA 0.5 µg/L, and HA 0.5 µg/L treated crayfish exhibited peak behavioral deficits on trial days eight to ten (DEA 10 µg/L) and trial day twelve (DIA 0.5 µg/L and HA 0.5 µg/L). Such results provide evidence that the detrimental toxic effects of ATR 3 µg/L, DEA 10 µg/L, DIA 0.5 µg/L, and HA 0.5 µg/L became more pronounced with prolonged exposure.

The behavioral deficits observed in crayfish as a result of exposure to atrazine and its three primary environmental degradation products included increased lethargy and impaired boldness and explorative behavior. Such impairments in behavior are likely to have profound effects on animals' abilities to survive within the environment. Crayfish exposed to atrazine,

DEA, DIA and HA will have impaired abilities to locate food, avoid predation, and locate potential mates. Considering crayfish are the third most endangered faunal group in North America (43% are ranked as imperiled), as well as within the world (Cordeiro, 2010) these implications are particularly relevant. The implications of these results also extend beyond *P. clarkii* to include any other species that depend on crayfish directly as a food source. Burrowing crayfish are also vital in wetland habitats such as swamps, wet fields, and marshes as a result of their roles in creating habitats upon which other species have coevolved to rely (Ernst & Ernst, 2003; Pintor & Soluk, 2006). Such burrows also prevent soil compaction by aerating soils (Welch *et al*., 2008). Furthermore, crayfish are keystone species (Gutiérrez-Yurrita & Montes, 1999) that provide vital energy to their ecosystems via their roles as detritus shredders. Thus, even slight changes in crayfish populations due to environmental contaminants may have considerable repercussions on other species as well as on ecosystem as a whole. Therefore, the risks associated with the presence of atrazine and its degradates in the environment are considerable, and current levels considered safe by the EPA do pose a threat to crayfish, and likely many other organisms within aquatic environments.

DEA, DIA and HA were found to be comparably as toxic as the parent compound for some of the behavioral endpoints assessed. For example, most atrazine treatment groups, excluding ATR 0.5 µg/L, did not differ significantly from degradate treatment groups in latency to reward or reward arm. However, some trends in differences between the magnitudes of behavioral deficits produced by atrazine and its degradate treatment groups do allow one to predict an order of toxicity for these contaminants.

There is evidence that DIA is the least toxic of the chemicals tested, based on time to reward and time to reward arm data, although DIA was not significantly different from DEA,

DIA, and most ATR treatment groups for these response variables. However, both DIA treatment levels did differ significantly (less) in percent of crayfish to not locate reward arm compared to the other chemicals tested. DEA 0.5 µg/L was found to be significantly less toxic than both HA treatment groups and most atrazine groups (excluding ATR 3 µg/L) for percent of crayfish to not locate reward arm. Additionally, results indicate that DEA was significantly more toxic at the higher concentration, i.e. DEA 0.5 µg/L < DEA 10 µg/L. Furthermore, depending on which concentrations were being analyzed, atrazine was found to be less than or equally as toxic as both treatment levels of HA and DEA 10 µg/L. Interestingly, comparisons to atrazine concentrations of 3 µg/L or 200 µg/L were the only levels at which degradatse were found to be more toxic than the parent compound. However, if one compares these contaminants only at equal concentrations, i.e. 0.5 or 10 µg/L, results indicate that atrazine is comparably as toxic as HA (at both treatment levels) and DEA 10 µg/L. Additionally, atrazine 0.5 µg/L treatment group consistently exhibited the most significant behavioral deficits compared to control for most analyses (excluding turnaround times). Therefore, the proposed order of toxicity for atrazine and its three predominant environmental degradates is as follows: ATR ~ HA ≥ DEA > DIA.

Another intriguing observation of this study was the nonlinear dose-response observed for atrazine. For example, the amount of time spent under cover by atrazine treatment groups was ATR 200 µg/L < ATR 3 µg/L < ATR 10 µg/L < ATR 0.5 µg/L (symbols here do not necessarily indicate significance). Many of the behavioral deficits observed for atrazine treatment groups in this research exhibit what the literature oftentimes refers to as a nonmonotonic dose-response curve (Vandenberg *et al.*, 2012; Flynn & Spellman, 2009). A dose-response curve is considered to be nonmonotonic when the slope of the curve changes direction at some point within the range of doses tested (Vandenberg *et al.*, 2012). Low dose effects and

nonmonotonic dose-responses occur often in studies of hormones and endocrine disrupting chemicals (EDCs), such as atrazine (Vandenberg *et al*., 2012; Greenman *et al*., 1997)

Toxicant induced disturbances within the neuroendocrine system have been shown to exist in conjunction with behavioral changes in numerous animal studies (Lin *et al.*, 2013; Rodriguez *et al*., 2013; Alvarez & Fuiman, 2005). This is not surprising, as neurotransmitters and hormones are known to moderate a suite of metabolic, developmental and behavioral pathways (Brown & Bern, 1989). Animal endocrine systems have evolved to respond to incredibly low levels of hormone, permitting numerous hormonal signaling agents to coexist and circulate (Welshons *et al.*, 2003). Hormones are capable of acting at such low concentrations for numerous reasons, including the following: receptors that are specific for a particular hormone have high affinity, thus they easily bind a sufficient amount of molecules to trigger a response, a nonlinear relationship exists between concentration of hormone and the amount of bound receptors, and there is a nonlinear relationship between the number of receptors that are bound and the greatest observable biologic effect (Vandenberg *et al.*, 2012).

Arthropods, including crayfish, have an open circulatory system, which bathes all internal organ system in hemolymph (Brusca & Brusca, 2003). Due to the circulatory system's organization, crustacean neuroendocrine structures are defined as any portions of the nervous system which contain secretory nerve terminals in direct contact with the hemolymph (Christie, 2011). Crayfish use a wide range of molecules as neurohormonal signaling agents. The largest and most diverse class of hormones is peptides, such as crustacean hyperglycemic hormone, or CHH (involved in carbohydrate metabolisms), A-type allatostatins (function as inhibitory neuro/myomodulators), and enkaphalins (regulate release of CHH) (Christie *et al*., 2010). Biogenic amines, including serotonin, octopamine and dopamine, often modulate other

hormones and neuromuscular transmission, have modulatory roles in control of neural circuit activity, function in the control of osmoregulation, and are cardio/vasoactive (Christie, 2011). More recently diffusible gases, particularly nitric oxide, have also been identified as potential signaling agents in crustaceans (Christie, 2011).

Due to shared receptor mediated mechanisms, EDCs, such as atrazine (and likely DEA, DIA and HA) that mimic naturally occurring hormones have been suggested to function in a similar manner as hormones and are thus able to induce low dose biological effects. Additionally, endocrine disrupting chemicals that affect in any manner hormone production, metabolism, release, or uptake will likely have effects at low, environmentally realistic doses, as even slight changes in concentration of hormone(s) can have biologically relevant effects (Vandenberg *et al.*, 2012; Welshons *et al.*, 2003).

Effects of EDCs and hormones are reliant upon dose, particularly low doses (such as within the physiological or environmental range). Such low doses are often more effective than high, toxicological doses at changing some endpoints (Vandenberg *et al*., 2012). Various mechanisms have been identified that explain how hormones and EDCs may produce nonmonotonic dose-responses in cells, tissues, and organisms.

Nonmonotonic dose-responses may be created by the combination of various monotonic responses that overlap and affect a common endpoint in opposite ways via differing pathways (Vandenberg *et al*., 2012). Such responses may also occur due to differences in receptor affinity, i.e. selectivity of the response, at high versus low doses. For example, at low doses an EDC might exclusively bind one type of receptor, but at higher doses it may also bind to other

hormone receptors. Various EDCs that have effects at environmentally realistic, low doses are known to act via multiple receptors and pathways (Vandeneberg *et al.,* 2012).

Receptors may also be down-regulated, resulting in a nonmonotnic dose-response (Vandeneberg *et al.,* 2012). Once a receptor is bound by a ligand, an elevated response is observed. As mentioned previously, nonlinear relationships exist between concentration of hormone and the amount of bound receptors and also between the amount of bound receptors and the biological effect (Welshons *et al.,* 2003). Once hormone binds a nuclear receptor and transcription of the target genes has taken place, the reaction must ultimately end and the receptor will eventually be inactivated in some manner (Vandenberg *et al*., 2012). As the level of hormone, or plausibly EDC, increases, the amount of inactivated and degraded receptors likewise increases; ultimately the amount of receptors being produced are unable to maintain the pace of the degradation pathway (Ismail & Nawaz, 2005). Additionally, the production of receptors is also affected by receptor internalization and degradation, which results in a more robust down-regulation of receptor (Modrall *et al.,* 2001). In organisms, receptor down-regulation roles are complex, as signaling from one hormone receptor may influence protein levels of a different receptor (Kinyamu & Archer, 2003).

Receptors may also be desensitized, a process by which a drop in response to a hormone is not a result of a decrease in the amount of available receptors, but is instead a result of the biochemical inactivation of a receptor (Freedman & Lefkowitz, 1996). This process usually occurs when continuous exposure to ligand takes places, and is normally observed with membrane-bound G protein-coupled receptors. Receptor activation, which occurs once a ligand binds, is very quickly followed by uncoupling of the activated receptor from the G proteins as a result of phosphorylation (Lohse, 1993). It is noteworthy that desensitization and down-

regulation can both take place in the same cells for the same receptor (Shankaran *et al*., 2007); thus, both can function to produce a nonmonotonic dose-response.

It has been suggested by mathematical modeling studies (Kohn & Melnick, 2002) that the combination of naturally occurring hormones and EDCs in the presence of unoccupied hormone receptors leads to some of the unoccupied receptors being bound by EDC, which may lead to an elevation in biological responses, such as increased organ weight or the elevated expression of a responsive gene (Vandenberg *et al*., 2012). At lower concentrations, both hormones and EDCs may bind and thus activate such a response; however, at higher doses, the EDC may outcompete hormones for receptor binding (Vandenberg *et al*., 2012).

Negative feedback loops exist in endocrine systems and may also contribute to the production of nonmonotonic does-response curves. Oftentimes, the synthesis of hormones is regulated by several positive, as well as negative feedback loops (Vandenberg *et al.,* 2012). Numerous hormones are known to influence or control their own secretion via such feedback systems. For example, insulin levels regulate the uptake of glucose by cells, and glucose levels promote insulin production. As glucose is removed from circulation, due to insulin, insulin levels decline (Vandenberg *et al*., 2012). Therefore, nonmonotonic dose responses are able to take place as the available ligand and receptor levels are moderated by each other, and it is plausible that EDCs may function in a similar fashion influencing levels of both endogenous hormones and receptors (Vandenberg *et al*., 2012).

Furthermore, studies of cultured cells have suggested that different gene profiles are affected by different levels of hormones. For example, in a study of genes affected by low versus higher estrogen doses, scientists discovered that a small number of genes in MCF7 breast cancer

cells were highly sensitive to low doses, 10 pM, of estradiol compared to the total number of genes that were influenced by higher, 30 or 100 pM, levels (Coser *et al*., 2003). Interestingly, the amount of estradiol-suppressed genes, as a result of 10 pM exposure, was about three times greater than the amount of estradiol-induced genes; however, the overall number of estradiol-suppressible genes was roughly half the total amount of estradiol-inducible genes (Coser *et al*., 2003). This research implies that low levels of estrogen selectively target a small portion of the overall number of estrogen-sensitive genes, and furthermore, the affected genes are likely to be suppressed by low level estrogen treatment (Vandenberg *et al*., 2012). Thus, nonmonotonic dose-response curves, due to the presence of hormones and/or EDCs, may also result from the pattern of gene expression.

In conclusion, exposure to atrazine, DEA, DIA and HA at low, environmentally realistic doses resulted in various behavioral deficits in *P. clarkii* that will likely affect this species ability to survive in natural environments. As explained previously, these implications extend beyond the lab, and the risks associated with the presence of atrazine and its degradation products in the environment are likely to include greater ecological repercussions. Furthermore, it is suggested that the behavioral deficits observed may have been modulated via contaminants' interference with the neuroendocrine system of crayfish, although this was not tested directly in this study. EDCs are known to alter animal behavior (Lin *et al.,* 2013; Rodriguez *et al*., 2013; Alvarez & Fuiman, 2005). Atrazine is a known endocrine disruptor (Hayes *et al*., 2011), and it is quite plausible that its degradates are as well, based on the observation that each of the contaminants tested caused similar and significant behavioral deficits in the model organism.

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Appendix

Table. A.1. Number of crayfish (n) per trial for each atrazine, DEA, DIA and HA treatment group. n varied throughout the trial period due to technical difficulties and/or experimenter error. Treatments were in µg/L.

Treatment	**Trial (Day)**														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Control	16	17	17	17	17	15	17	17	17	17	17	16	16	17	17
ATR 0.5	10	11	10	11	10	11	10	10	11	11	11	11	11	11	11
ATR 3	11	12	12	12	11	11	12	11	12	12	12	12	11	12	10
ATR 10	10	10	9	10	10	9	10	10	9	10	10	10	9	10	7
ATR 200	12	12	12	12	11	8	9	11	11	11	9	9	10	9	9
DEA 0.5	11	10	11	10	11	11	11	10	11	11	9	6	11	10	11
DEA 10	11	11	11	11	11	11	11	11	11	11	11	10	10	11	7
DIA 0.5	10	9	10	11	11	11	11	9	11	11	9	5	11	11	11
DIA 10	11	11	11	11	10	10	9	9	11	10	11	11	11	11	8
HA 0.5	11	10	10	11	11	11	11	8	11	11	11	6	10	11	11
HA 10	11	11	11	11	11	10	10	10	10	11	11	11	11	11	9

Table. A.2. Time to Reward Differences of Treatment Least Square Means (Alpha = 0.05). * in right hand column represents a significant difference between treatments in columns on far left.

Treatment	_Treatment	Estimate	Standard Error	p-value	Lower	Upper	
ATR 200	HA 10	-0.2593	0.2329	0.2657	-0.716	0.1975	
ATR 200	HA 0.5	-0.2394	0.233	0.3043	-0.6963	0.2175	
ATR 200	ATR 10	-0.1004	0.239	0.6744	-0.5693	0.3684	
ATR 200	ATR 3	-0.1142	0.2279	0.6164	-0.5611	0.3327	
ATR 200	ATR 0.5	-0.3353	0.2327	0.1498	-0.7917	0.1211	
ATR 200	DEA 10	-0.04575	0.2335	0.8447	-0.5037	0.4122	
ATR 200	DEA 0.5	0.02263	0.2333	0.9227	-0.435	0.4802	
ATR 200	DIA 10	0.1189	0.2334	0.6106	-0.3389	0.5767	
ATR 200	DIA 0.5	0.171	0.2339	0.4649	-0.2878	0.6297	
ATR 200	Control	0.4755	0.2114	0.0246	0.06087	0.8901	*
HA 10	HA 0.5	0.01988	0.2366	0.9331	-0.4442	0.484	
HA 10	ATR 10	0.1588	0.2426	0.5128	-0.317	0.6347	
HA 10	ATR 3	0.1451	0.2316	0.5311	-0.3092	0.5994	
HA 10	ATR 0.5	-0.07602	0.2364	0.7478	-0.5396	0.3876	
HA 10	DEA 10	0.2135	0.2372	0.3682	-0.2517	0.6787	
HA 10	DEA 0.5	0.2819	0.237	0.2344	-0.1829	0.7467	
HA 10	DIA 10	0.3782	0.2371	0.1109	-0.0868	0.8431	
HA 10	DIA 0.5	0.4302	0.2376	0.0703	-0.0357	0.8962	
HA 10	Control	0.7347	0.2154	0.0007	0.3122	1.1573	*
HA 0.5	ATR 10	0.1389	0.2427	0.5671	-0.3371	0.615	
HA 0.5	ATR 3	0.1252	0.2317	0.589	-0.3292	0.5797	
HA 0.5	ATR 0.5	-0.0959	0.2365	0.6851	-0.5597	0.3679	
HA 0.5	DEA 10	0.1936	0.2373	0.4146	-0.2718	0.659	
HA 0.5	DEA 0.5	0.262	0.2371	0.2692	-0.2029	0.727	
HA 0.5	DIA 10	0.3583	0.2372	0.131	-0.1069	0.8234	
HA 0.5	DIA 0.5	0.4103	0.2376	0.0844	-0.0558	0.8764	
HA 0.5	Control	0.7149	0.2155	0.0009	0.2921	1.1376	*
ATR 10	ATR 3	-0.01372	0.2378	0.954	-0.4801	0.4527	
ATR 10	ATR 0.5	-0.2348	0.2424	0.3329	-0.7104	0.2407	
ATR 10	DEA 10	0.05469	0.2432	0.8221	-0.4224	0.5318	
ATR 10	DEA 0.5	0.1231	0.243	0.6126	-0.3536	0.5997	
ATR 10	DIA 10	0.2193	0.2431	0.3671	-0.2575	0.6962	
ATR 10	DIA 0.5	0.2714	0.2436	0.2654	-0.2064	0.7492	
ATR 10	Control	0.5759	0.2221	0.0096	0.1403	1.0115	*

ATR 3	ATR 0.5	-0.2211	0.2314	0.3395	-0.6751	0.2328	
ATR 3	DEA 10	0.06842	0.2323	0.7684	-0.3872	0.524	
ATR 3	DEA 0.5	0.1368	0.232	0.5556	-0.3183	0.5919	
ATR 3	DIA 10	0.2331	0.2321	0.3156	-0.2223	0.6884	
ATR 3	DIA 0.5	0.2851	0.2326	0.2205	-0.1712	0.7414	
ATR 3	Control	0.5896	0.21	0.005	0.1777	1.0015	*
ATR 0.5	DEA 10	0.2895	0.237	0.2221	-0.1754	0.7544	
ATR 0.5	DEA 0.5	0.3579	0.2368	0.1309	-0.1065	0.8224	
ATR 0.5	DIA 10	0.4542	0.2369	0.0554	-0.0105	0.9188	
ATR 0.5	DIA 0.5	0.5062	0.2374	0.0331	0.04064	0.9718	*
ATR 0.5	Control	0.8108	0.2152	0.0002	0.3886	1.2329	*
DEA 10	DEA 0.5	0.06838	0.2376	0.7736	-0.3977	0.5345	
DEA 10	DIA 10	0.1646	0.2377	0.4887	-0.3016	0.6309	
DEA 10	DIA 0.5	0.2167	0.2382	0.363	-0.2505	0.6839	
DEA 10	Control	0.5212	0.2161	0.016	0.0973	0.9451	*
DEA 0.5	DIA 10	0.09626	0.2375	0.6853	-0.3696	0.5621	
DEA 0.5	DIA 0.5	0.1483	0.238	0.5332	-0.3184	0.6151	
DEA 0.5	Control	0.4528	0.2159	0.0361	0.02937	0.8763	*
DIA 10	DIA 0.5	0.05207	0.2381	0.8269	-0.4149	0.519	
DIA 10	Control	0.3566	0.216	0.099	-0.0671	0.7803	
DIA 0.5	Control	0.3045	0.2165	0.1598	-0.1202	0.7292	

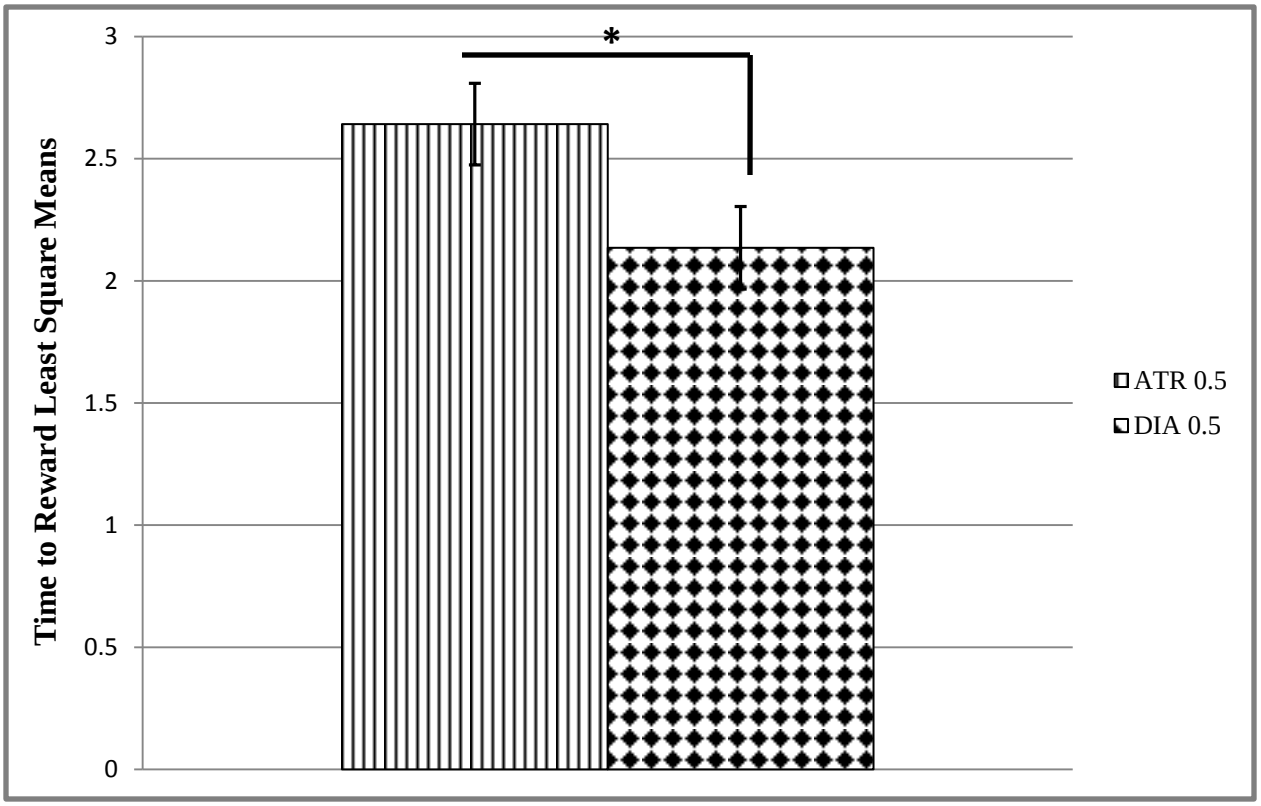

Fig. A.1. Time to reward differences of LSMeans comparing atrazine 0.5 ug/L and DIA 0.5 ug/L. Based on pairwise comparisons (see Table A.2), these were the only atrazine and degradate treatment groups that differed significantly. Results indicate that atrazine is significantly more toxic than DIA at the lowest concentration tested.

Table A.3. Time to Reward Arm Least Differences of Least Square Means (Alpha = 0.05). * in right hand column represents a significant difference between treatments in columns on far left.

Treatment	_Treatment	Estimate	Standard Error	p-value	Lower	Upper	
ATR 200	HA 10	-0.2949	0.2573	0.252	-0.7996	0.2098	
ATR 200	HA 0.5	-0.286	0.2574	0.2667	-0.7908	0.2188	
ATR 200	ATR 10	-0.06751	0.2642	0.7983	-0.5857	0.4507	
ATR 200	ATR 3	-0.169	0.2517	0.5022	-0.6626	0.3247	
ATR 200	ATR 0.5	-0.4265	0.257	0.0973	-0.9306	0.0777	
ATR 200	DEA 10	-0.1708	0.2579	0.5079	-0.6765	0.335	
ATR 200	DEA 0.5	0.002418	0.2576	0.9925	-0.5029	0.5078	
ATR 200	DIA 10	0.06915	0.2578	0.7886	-0.4365	0.5748	
ATR 200	DIA 0.5	0.08587	0.2582	0.7395	-0.4206	0.5923	
ATR 200	Control	0.4733	0.2336	0.0429	0.01518	0.9314	*
HA 10	HA 0.5	0.008895	0.2615	0.9729	-0.504	0.5218	
HA 10	ATR 10	0.2274	0.2682	0.3967	-0.2987	0.7534	
HA 10	ATR 3	0.1259	0.2559	0.6228	-0.376	0.6278	
HA 10	ATR 0.5	-0.1316	0.2612	0.6144	-0.6438	0.3807	
HA 10	DEA 10	0.1241	0.262	0.6358	-0.3898	0.638	
HA 10	DEA 0.5	0.2973	0.2618	0.2562	-0.2161	0.8107	
HA 10	DIA 10	0.364	0.2619	0.1648	-0.1497	0.8777	
HA 10	DIA 0.5	0.3807	0.2623	0.1468	-0.1337	0.8952	
HA 10	Control	0.7682	0.2381	0.0013	0.3011	1.2352	*
HA 0.5	ATR 10	0.2185	0.2682	0.4155	-0.3077	0.7446	
HA 0.5	ATR 3	0.117	0.2559	0.6476	-0.385	0.619	
HA 0.5	ATR 0.5	-0.1405	0.2612	0.5908	-0.6528	0.3718	
HA 0.5	DEA 10	0.1152	0.262	0.6602	-0.3987	0.6292	
HA 0.5	DEA 0.5	0.2884	0.2618	0.2708	-0.2251	0.8019	
HA 0.5	DIA 10	0.3551	0.262	0.1754	-0.1587	0.8689	
HA 0.5	DIA 0.5	0.3718	0.2623	0.1566	-0.1427	0.8864	
HA 0.5	Control	0.7593	0.2382	0.0015	0.2922	1.2264	*
ATR 10	ATR 3	-0.1014	0.2628	0.6996	-0.6169	0.414	
ATR 10	ATR 0.5	-0.359	0.2679	0.1805	-0.8845	0.1666	
ATR 10	DEA 10	-0.1032	0.2687	0.7009	-0.6303	0.4238	
ATR 10	DEA 0.5	0.06993	0.2685	0.7946	-0.4567	0.5966	
ATR 10	DIA 10	0.1367	0.2687	0.611	-0.3903	0.6636	
ATR 10	DIA 0.5	0.1534	0.269	0.5687	-0.3743	0.6811	
ATR 10	Control	0.5408	0.2455	0.0277	0.05928	1.0223	*
ATR 3	ATR 0.5	-0.2575	0.2556	0.3139	-0.7589	0.2438	

ATR 3	DEA 10	-0.00181	0.2565	0.9944	-0.5048	0.5012	
ATR 3	DEA 0.5	0.1714	0.2562	0.5037	-0.3312	0.6739	
ATR 3	DIA 10	0.2381	0.2564	0.3532	-0.2648	0.741	
ATR 3	DIA 0.5	0.2548	0.2568	0.3212	-0.2488	0.7585	
ATR 3	Control	0.6423	0.232	0.0057	0.1872	1.0973	*
ATR 0.5	DEA 10	0.2557	0.2617	0.3287	-0.2576	0.769	
ATR 0.5	DEA 0.5	0.4289	0.2615	0.1012	-0.084	0.9417	
ATR 0.5	DIA 10	0.4956	0.2616	0.0584	-0.0176	1.0088	
ATR 0.5	DIA 0.5	0.5123	0.262	0.0507	-0.0016	1.0263	
ATR 0.5	Control	0.8998	0.2378	0.0002	0.4333	1.3662	*
DEA 10	DEA 0.5	0.1732	0.2623	0.5092	-0.3413	0.6877	
DEA 10	DIA 10	0.2399	0.2625	0.3608	-0.2749	0.7547	
DEA 10	DIA 0.5	0.2566	0.2628	0.329	-0.2589	0.7722	
DEA 10	Control	0.6441	0.2387	0.007	0.1759	1.1122	*
DEA 0.5	DIA 10	0.06673	0.2622	0.7992	-0.4476	0.5811	
DEA 0.5	DIA 0.5	0.08345	0.2626	0.7507	-0.4316	0.5985	
DEA 0.5	Control	0.4709	0.2385	0.0485	0.00317	0.9386	*
DIA 10	DIA 0.5	0.01672	0.2628	0.9493	-0.4987	0.5321	
DIA 10	Control	0.4042	0.2386	0.0905	-0.0639	0.8722	
DIA 0.5	Control	0.3874	0.2391	0.1053	-0.0815	0.8563	

Table A.4. Percent of Crayfish Not to Locate Reward Arm Differences of Treatment Least Square Means (Alpha = 0.05). * in right hand column represents a significant difference between treatments in columns on far left.

Treatment	_Treatment	Estimate	Standard Error	p-value	Lower	Upper	
ATR 200	HA 10	-0.03231	0.3787	0.9321	-0.7805	0.7159	
ATR 200	HA 0.5	0.277	0.3802	0.4674	-0.4741	1.0281	
ATR 200	ATR 10	0.4377	0.3812	0.2526	-0.3153	1.1908	
ATR 200	ATR 3	0.9301	0.3853	0.017	0.1689	1.6913	*
ATR 200	ATR 0.5	-0.1623	0.3782	0.6685	-0.9095	0.5849	
ATR 200	DEA 10	0.01405	0.3789	0.9705	-0.7345	0.7626	
ATR 200	DEA 0.5	1.5587	0.3945	0.0001	0.7795	2.338	*
ATR 200	DIA 10	1.8799	0.4017	<.0001	1.0863	2.6735	*
ATR 200	DIA 0.5	1.0982	0.3872	0.0052	0.3332	1.8632	*
ATR 200	Control	1.676	0.3969	<.0001	0.892	2.46	*
HA 10	HA 0.5	0.3093	0.3801	0.417	-0.4416	1.0601	
HA 10	ATR 10	0.4701	0.381	0.2192	-0.2827	1.2228	
HA 10	ATR 3	0.9624	0.3852	0.0135	0.2015	1.7234	*
HA 10	ATR 0.5	-0.13	0.3781	0.7315	-0.8769	0.6169	
HA 10	DEA 10	0.04636	0.3788	0.9027	-0.7019	0.7946	
HA 10	DEA 0.5	1.5911	0.3943	<.0001	0.8121	2.37	*
HA 10	DIA 10	1.9122	0.4016	<.0001	1.1189	2.7056	*
HA 10	DIA 0.5	1.1305	0.3871	0.004	0.3658	1.8953	*
HA 10	Control	1.7083	0.3967	<.0001	0.9246	2.4921	*
HA 0.5	ATR 10	0.1608	0.3825	0.6749	-0.5949	0.9164	
HA 0.5	ATR 3	0.6532	0.3866	0.0932	-0.1107	1.417	
HA 0.5	ATR 0.5	-0.4393	0.3796	0.249	-1.1891	0.3106	
HA 0.5	DEA 10	-0.2629	0.3803	0.4904	-1.0141	0.4883	
HA 0.5	DEA 0.5	1.2818	0.3958	0.0015	0.5	2.0636	*
HA 0.5	DIA 10	1.6029	0.403	0.0001	0.8068	2.3991	*
HA 0.5	DIA 0.5	0.8213	0.3886	0.0362	0.05365	1.5889	*
HA 0.5	Control	1.3991	0.3982	0.0006	0.6125	2.1856	*
ATR 10	ATR 3	0.4924	0.3876	0.2059	-0.2733	1.2581	
ATR 10	ATR 0.5	-0.6	0.3806	0.1169	-1.3518	0.1517	
ATR 10	DEA 10	-0.4237	0.3812	0.2681	-1.1768	0.3294	
ATR 10	DEA 0.5	1.121	0.3967	0.0053	0.3373	1.9047	*
ATR 10	DIA 10	1.4422	0.4039	0.0005	0.6442	2.2401	*
ATR 10	DIA 0.5	0.6605	0.3895	0.092	-0.109	1.43	
ATR 10	Control	1.2383	0.3991	0.0023	0.4499	2.0267	*
ATR 3	ATR 0.5	-1.0924	0.3847	0.0051	-1.8524	-0.3325	*

ATR 3	DEA 10	-0.9161	0.3854	0.0187	-1.6774	-0.1548	*
ATR 3	DEA 0.5	0.6286	0.4007	0.1187	-0.1629	1.4201	
ATR 3	DIA 10	0.9498	0.4078	0.0212	0.1441	1.7554	*
ATR 3	DIA 0.5	0.1681	0.3936	0.6699	-0.6094	0.9456	
ATR 3	Control	0.7459	0.403	0.0661	-0.0503	1.5421	
ATR 0.5	DEA 10	0.1763	0.3783	0.6418	-0.571	0.9236	
ATR 0.5	DEA 0.5	1.721	0.3938	<.0001	0.943	2.4991	*
ATR 0.5	DIA 10	2.0422	0.4011	<.0001	1.2498	2.8346	*
ATR 0.5	DIA 0.5	1.2605	0.3866	0.0014	0.4968	2.0243	*
ATR 0.5	Control	1.8383	0.3963	<.0001	1.0555	2.6211	*
DEA 10	DEA 0.5	1.5447	0.3945	0.0001	0.7653	2.3241	*
DEA 10	DIA 10	1.8659	0.4018	<.0001	1.0721	2.6596	*
DEA 10	DIA 0.5	1.0842	0.3873	0.0058	0.3191	1.8493	*
DEA 10	Control	1.662	0.3969	<.0001	0.8779	2.4461	*
DEA 0.5	DIA 10	0.3212	0.4165	0.4418	-0.5016	1.1439	
DEA 0.5	DIA 0.5	-0.4605	0.4025	0.2544	-1.2557	0.3347	
DEA 0.5	Control	0.1173	0.4118	0.7762	-0.6962	0.9307	
DIA 10	DIA 0.5	-0.7817	0.4096	0.0582	-1.5909	0.02758	
DIA 10	Control	-0.2039	0.4187	0.627	-1.0311	0.6233	
DIA 0.5	Control	0.5778	0.4049	0.1556	-0.222	1.3776	

Table A.5. Under Cover Differences of Treatment Least Square Means (Alpha = 0.05). * in right hand column represents a significant difference between treatments in columns on far left.

Treatment	_Treatment	Estimate	Standard Error	p-value	Lower	Upper	
ATR 200	HA 10	-0.9351	0.4279	0.029	-1.7744	-0.0957	*
ATR 200	HA 0.5	-0.9804	0.427	0.0218	-1.8178	-0.1429	*
ATR 200	ATR 10	-0.9007	0.438	0.0399	-1.7597	-0.0418	*
ATR 200	ATR 3	-0.5347	0.4183	0.2013	-1.3551	0.2857	
ATR 200	ATR 0.5	-1.2843	0.4265	0.0026	-2.1208	-0.4478	*
ATR 200	DEA 10	-0.8791	0.4293	0.0408	-1.7212	-0.037	*
ATR 200	DEA 0.5	-0.3497	0.4282	0.4141	-1.1895	0.49	
ATR 200	DIA 10	-0.5023	0.428	0.2408	-1.3417	0.3372	
ATR 200	DIA 0.5	-0.5061	0.4294	0.2387	-1.3484	0.3361	
ATR 200	Control	0.689	0.3933	0.08	-0.0824	1.4604	
HA 10	HA 0.5	-0.0453	0.4317	0.9164	-0.8921	0.8015	
HA 10	ATR 10	0.03433	0.4426	0.9382	-0.8338	0.9024	
HA 10	ATR 3	0.4004	0.4231	0.3441	-0.4295	1.2303	
HA 10	ATR 0.5	-0.3492	0.4312	0.4182	-1.195	0.4966	
HA 10	DEA 10	0.05597	0.4342	0.8974	-0.7956	0.9075	
HA 10	DEA 0.5	0.5853	0.4329	0.1765	-0.2638	1.4344	
HA 10	DIA 10	0.4328	0.4328	0.3174	-0.416	1.2816	
HA 10	DIA 0.5	0.4289	0.4342	0.3234	-0.4227	1.2805	
HA 10	Control	1.6241	0.3986	<.0001	0.8423	2.4059	*
HA 0.5	ATR 10	0.07963	0.4416	0.8569	-0.7865	0.9458	
HA 0.5	ATR 3	0.4457	0.4221	0.2911	-0.3821	1.2736	
HA 0.5	ATR 0.5	-0.3039	0.4302	0.4801	-1.1477	0.5399	
HA 0.5	DEA 10	0.1013	0.4333	0.8152	-0.7485	0.951	
HA 0.5	DEA 0.5	0.6306	0.4319	0.1444	-0.2165	1.4777	
HA 0.5	DIA 10	0.4781	0.4317	0.2683	-0.3687	1.3249	
HA 0.5	DIA 0.5	0.4742	0.4332	0.2738	-0.3755	1.324	
HA 0.5	Control	1.6694	0.3976	<.0001	0.8894	2.4493	*
ATR 10	ATR 3	0.3661	0.4332	0.3982	-0.4835	1.2157	
ATR 10	ATR 0.5	-0.3835	0.4411	0.3847	-1.2487	0.4817	
ATR 10	DEA 10	0.02164	0.4441	0.9611	-0.8493	0.8926	
ATR 10	DEA 0.5	0.551	0.4427	0.2135	-0.3174	1.4194	
ATR 10	DIA 10	0.3985	0.4426	0.3681	-0.4696	1.2666	
ATR 10	DIA 0.5	0.3946	0.444	0.3743	-0.4763	1.2655	
ATR 10	Control	1.5897	0.4094	0.0001	0.7868	2.3927	*
ATR 3	ATR 0.5	-0.7496	0.4216	0.0756	-1.5765	0.07726	

ATR 3	DEA 10	-0.3444	0.4247	0.4175	-1.1774	0.4885	
ATR 3	DEA 0.5	0.1849	0.4233	0.6623	-0.6453	1.0151	
ATR 3	DIA 10	0.0324	0.4231	0.939	-0.7975	0.8623	
ATR 3	DIA 0.5	0.02852	0.4246	0.9465	-0.8044	0.8614	
ATR 3	Control	1.2237	0.3883	0.0017	0.4621	1.9853	*
ATR 0.5	DEA 10	0.4052	0.4328	0.3493	-0.4436	1.254	
ATR 0.5	DEA 0.5	0.9345	0.4314	0.0304	0.08838	1.7807	*
ATR 0.5	DIA 10	0.782	0.4312	0.07	-0.0638	1.6278	
ATR 0.5	DIA 0.5	0.7781	0.4327	0.0723	-0.0706	1.6269	
ATR 0.5	Control	1.9733	0.3971	<.0001	1.1944	2.7522	*
DEA 10	DEA 0.5	0.5293	0.4344	0.2232	-0.3227	1.3814	
DEA 10	DIA 10	0.3768	0.4343	0.3856	-0.4749	1.2286	
DEA 10	DIA 0.5	0.373	0.4356	0.392	-0.4814	1.2273	
DEA 10	Control	1.5681	0.4	<.0001	0.7836	2.3526	*
DEA 0.5	DIA 10	-0.1525	0.4329	0.7246	-1.0016	0.6966	
DEA 0.5	DIA 0.5	-0.1564	0.4344	0.7189	-1.0084	0.6956	
DEA 0.5	Control	1.0388	0.3989	0.0093	0.2563	1.8212	*
DIA 10	DIA 0.5	-0.00387	0.4342	0.9929	-0.8556	0.8478	
DIA 10	Control	1.1913	0.3987	0.0029	0.4092	1.9733	*
DIA 0.5	Control	1.1951	0.4002	0.0029	0.4103	1.98	*

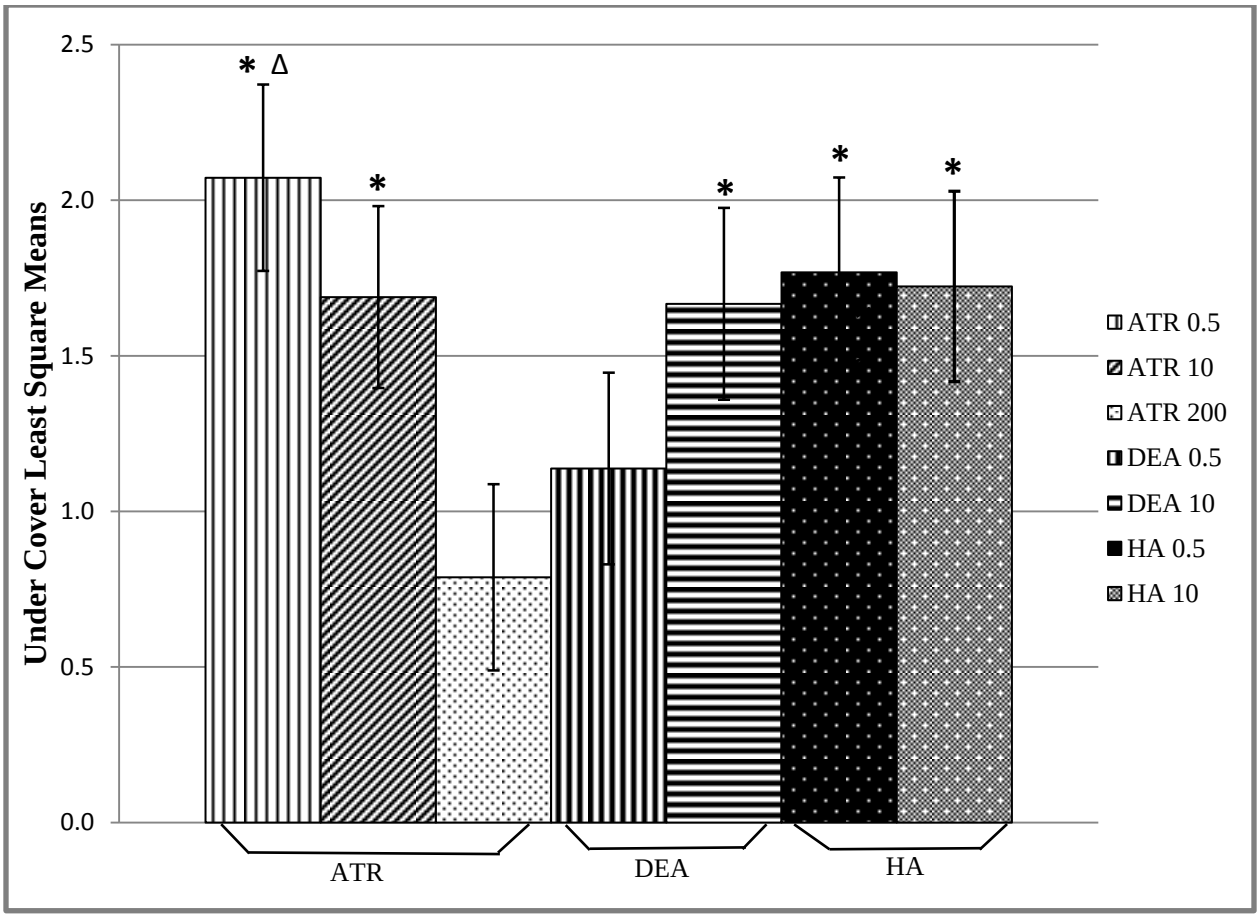

Fig. A.2. Time spent in under cover area of the maze LSMeans comparing atrazine and degradate treatment groups in pairwise fashion. P-values are listed in Table A.5.

- Represents treatment was significantly > ATR 200 : *****
- Represents treatment was significantly > DEA 0.5: Δ

Several treatments group spent significantly longer under cover compared to ATR 200, including: HA 0.5 &10, ATR 0.5 &10, and DEA 10. Also, ATR 0.5 was significantly greater than DEA 0.5 for under cover time. These results suggest that atrazine is more toxic at lower concentrations, with the most toxic level being 0.5 µg/L.

Table A.6. ~ 180 ° Turnaround Differences of Treatment Least Square Means (Alpha = 0.05). * in right hand column represents a significant difference between treatments in columns on far left.

Treatment	_Treatment	Estimate	Standard Error	p-Value	Lower	Upper	
ATR 200	HA 10	-0.1727	0.1143	0.1312	-0.397	0.05166	
ATR 200	HA 0.5	-0.2948	0.1112	0.0081	-0.513	-0.0767	*
ATR 200	ATR 10	-0.1741	0.1184	0.142	-0.4065	0.05836	
ATR 200	ATR 3	-0.05775	0.1085	0.5945	-0.2706	0.1551	
ATR 200	ATR 0.5	-0.2034	0.1146	0.0763	-0.4284	0.02158	
ATR 200	DEA 10	-0.2278	0.1122	0.0426	-0.4479	-0.0077	*
ATR 200	DEA 0.5	-0.2347	0.1101	0.0333	-0.4507	-0.0186	*
ATR 200	DIA 10	-0.2352	0.1087	0.0307	-0.4485	-0.0219	*
ATR 200	DIA 0.5	-0.1287	0.1153	0.2646	-0.3551	0.0976	
ATR 200	Control	0.4112	0.1004	<.0001	0.2142	0.6083	*
HA 10	HA 0.5	-0.1222	0.1175	0.2989	-0.3529	0.1085	
HA 10	ATR 10	-0.00143	0.1244	0.9908	-0.2456	0.2428	
HA 10	ATR 3	0.1149	0.115	0.318	-0.1108	0.3406	
HA 10	ATR 0.5	-0.03074	0.1208	0.7993	-0.2679	0.2064	
HA 10	DEA 10	-0.05513	0.1185	0.6418	-0.2877	0.1774	
HA 10	DEA 0.5	-0.062	0.1165	0.5948	-0.2907	0.1667	
HA 10	DIA 10	-0.06256	0.1152	0.5872	-0.2887	0.1635	
HA 10	DIA 0.5	0.04392	0.1215	0.7177	-0.1944	0.2823	
HA 10	Control	0.5839	0.1074	<.0001	0.3731	0.7947	*
HA 0.5	ATR 10	0.1207	0.1216	0.3209	-0.1179	0.3594	
HA 0.5	ATR 3	0.2371	0.1118	0.0342	0.01774	0.4564	*
HA 0.5	ATR 0.5	0.09145	0.1178	0.4376	-0.1397	0.3225	
HA 0.5	DEA 10	0.06705	0.1154	0.5615	-0.1595	0.2936	
HA 0.5	DEA 0.5	0.06018	0.1134	0.5958	-0.1624	0.2827	
HA 0.5	DIA 10	0.05962	0.1121	0.5949	-0.1604	0.2796	
HA 0.5	DIA 0.5	0.1661	0.1185	0.1614	-0.0665	0.3987	
HA 0.5	Control	0.7061	0.1041	<.0001	0.5018	0.9104	*
ATR 10	ATR 3	0.1163	0.1191	0.3291	-0.1175	0.3501	
ATR 10	ATR 0.5	-0.0293	0.1248	0.8144	-0.2742	0.2156	
ATR 10	DEA 10	-0.0537	0.1225	0.6612	-0.2941	0.1867	
ATR 10	DEA 0.5	-0.06057	0.1206	0.6156	-0.2972	0.1761	
ATR 10	DIA 10	-0.06113	0.1193	0.6085	-0.2953	0.173	
ATR 10	DIA 0.5	0.04535	0.1253	0.7176	-0.2006	0.2913	
ATR 10	Control	0.5853	0.1118	<.0001	0.3659	0.8047	*
ATR 3	ATR 0.5	-0.1456	0.115	0.2059	-0.3714	0.08014	

ATR 3	DEA 10	-0.17	0.1128	0.1319	-0.3914	0.05128	
ATR 3	DEA 0.5	-0.1769	0.1107	0.1104	-0.3942	0.04035	
ATR 3	DIA 10	-0.1775	0.1094	0.1051	-0.3922	0.03722	
ATR 3	DIA 0.5	-0.07099	0.116	0.5406	-0.2986	0.1566	
ATR 3	Control	0.469	0.1012	<.0001	0.2703	0.6677	*
ATR 0.5	DEA 10	-0.0244	0.1187	0.8372	-0.2574	0.2086	
ATR 0.5	DEA 0.5	-0.03127	0.1167	0.7889	-0.2604	0.1979	
ATR 0.5	DIA 10	-0.03183	0.1155	0.783	-0.2585	0.1949	
ATR 0.5	DIA 0.5	0.07466	0.1217	0.5399	-0.1643	0.3136	
ATR 0.5	Control	0.6146	0.1078	<.0001	0.403	0.8262	*
DEA 10	DEA 0.5	-0.00687	0.1144	0.9521	-0.2314	0.2176	
DEA 10	DIA 10	-0.00743	0.1131	0.9476	-0.2294	0.2145	
DEA 10	DIA 0.5	0.09905	0.1195	0.4072	-0.1354	0.3335	
DEA 10	Control	0.639	0.1052	<.0001	0.4326	0.8454	*
DEA 0.5	DIA 10	-0.00056	0.111	0.996	-0.2184	0.2173	
DEA 0.5	DIA 0.5	0.1059	0.1175	0.3675	-0.1247	0.3365	
DEA 0.5	Control	0.6459	0.1029	<.0001	0.4439	0.8479	*
DIA 10	DIA 0.5	0.1065	0.1162	0.3597	-0.1216	0.3345	
DIA 10	Control	0.6465	0.1014	<.0001	0.4474	0.8455	*
DIA 0.5	Control	0.54	0.1085	<.0001	0.3271	0.7529	*

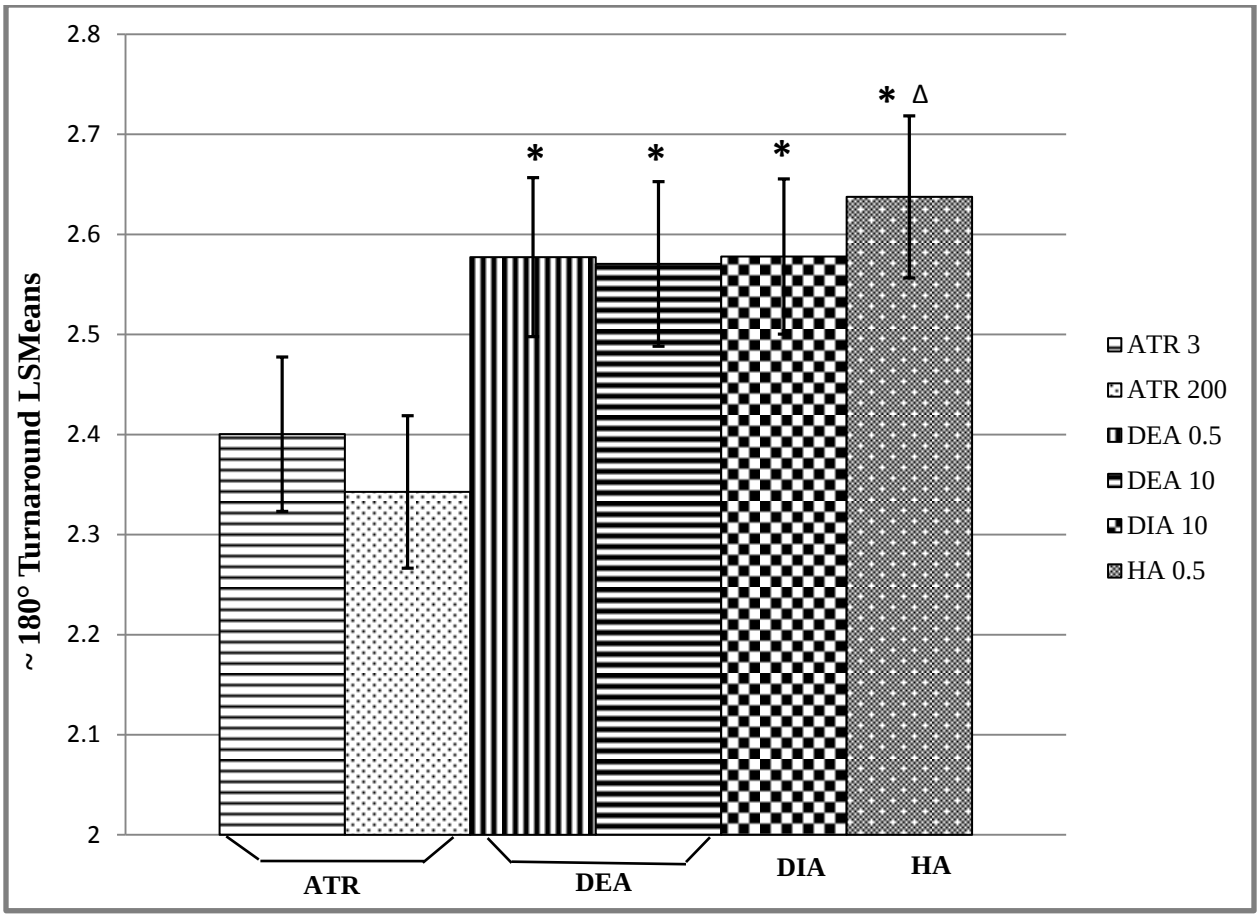

Fig. A.3. ~ 180° turnaround LSMeans comparing atrazine and degradate treatment groups in pairwise fashion. P-values are listed in Table A.6.

- Represents treatment was significantly > ATR 200 : *****
- Represents treatment was significantly > ATR 3: Δ

Several treatments group took significantly longer to perform turnarounds compared to ATR 200, including: HA 0.5, DEA 0.5 & 10, and DIA 10. Also, HA 0.5 took significantly longer than ATR 3 to perform turnarounds. Results indicate, once again, a nonlinear toxicity response for atrazine concentration.

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Office of Research Integrity

October 24, 2013

Carol B. Starkey
1340 15th Street Apt. 2
Huntington, WV 25701

Dear Ms. Starkey:

This letter is in response to the submitted thesis abstract entitled *"Subthreshold Toxic Effect of Atrazine and Three Degradates on Learning and Behavior in Procambarus Clarkii."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) Chair has also deemed this not to be animal research requiring their approval. The information in this study is not considered human subject or animal research as set forth in the definitions contained in the federal regulations. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director