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The Effects of Pharmaceutical and Estrone Exposure on Predator Avoidance Performance and Feeding Efficiency in Larval Fathead Minnows

Kyle Bird
biky0801@stcloudstate.edu

The Effects of Pharmaceutical and Estrone Exposure on Predator Avoidance Performance and Feeding Efficiency in Larval Fathead Minnows

By

Kyle Lee Bird

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Thesis Committee:

Heiko Schoenfuss, Chairperson

Timothy Schuh

Jessica Ward

Nathan Winter

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With the increased use of pharmaceuticals and endocrine active compounds, more of these chemical compounds are reaching the aquatic environment. Some pharmaceuticals and endocrine active compounds have reported effects concentrations in the nanogram per liter range. However, few experiments have examined the effects of these compounds on larval fish behavior. For the current study, fathead minnows *(Pimephales promelas*) were exposed to either an ethanol solvent control, diclofenac (1,600 ng L^{-1}), methocarbamol (4,800 ng L^{-1}), rosuvastatin (3,200 ng L^{-1}), sulfamethoxazole (2,200 ng L^{-1}), temazepam (1,600 ng L^{-1}), estrone (E1) at 125 ng L^{-1} or 625 ng L^{-1} for twenty-one days in a 50% daily static renewal system. The exposure began three days after fertilization of eggs when eye spots were observed. Following the twenty-one days of exposure, the larvae were subjected to a predator avoidance test to examine whether the pharmaceutical or E1 exposures caused a deficiency in the predator avoidance performance. Larvae exposed to rosuvastatin, diclofenac or 125 ng L^{-1} E1 exhibited a delayed latency period during the predator avoidance performance ($p<0.05$). A feeding efficiency test was also conducted to determine the effects of exposure on a larvae's ability to forage. Only larvae exposed to E1 (125 ng L^{-1}) showed deficiencies in the percentage of brine shrimp consumed. By examining a larvae's ability to effectively escape predation and to feed efficiently, two main aspects of evolutionary fitness were assessed. Our experiments demonstrated that both pharmaceuticals and E1 can diminish these behaviors. As a consequence, fish may fail to reach maturity, resulting in subsequent loss of reproduction with unknown population level consequences.

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Chapter I

LITERATURE REVIEW

Pharmaceuticals are widely used and have been detected in treated wastewater effluent that is discharged into waterways (Koplin, et al., 2002; Richardson, 2012; Schultz, et al., 2011). The persistence of pharmaceuticals in aquatic ecosystems is a result of their metabolic stability, making them less likely to be degraded during the wastewater treatment process (Dietrich, Webb, & Petry, 2002; Glassmeyer, et al., 2005). Those pharmaceuticals which remain metabolically active, have been observed ranging in concentrations from nanograms to micrograms per liter in surface waters and waste water effluent (Koplin, et al., 2002; Richardson, 2012; Ternes, 1998).

In addition to pharmaceuticals, steroidal and hormonal compounds, also known as endocrine active compounds (EACs), have been found in waste water effluents discharged into aquatic environments (Desbrow, Routledge, Brighty, Sumpter, & Waldock, 1998; Koplin, et al., 2002). A wide range of studies have examined how environmentally relevant concentrations of these compounds affect fathead minnows (Dammann, Shappell, Bartell, & Schoenfuss, 2011; Garcia-Reyero, et al., 2011; Hyndman, Biales, Bartell, & Schoenfuss, 2010; Kidd, et al., 2007; McGee, et al., 2009; Palace, et al., 2009; Schmid, Gonzalez-Valero, Rufli, & Dietrich, 2002; Shapell, Hyndman, Bartell, & Schoenfuss, 2010; Schoenfuss, Martinovic, & Sorenson, 2011; Thorpe, Benstead, Hutchinson, & Tyler, 2007). These studies highlight the physiological and biological changes that may result from EAC exposure. In studies by Kidd et al. (2007), and Palace et al. (2009), the synthetic steroidal hormone ethynlestradiol was added to a whole lake

and the authors observed a population decline in fathead minnows over two summers. Examples of exposures to EACs in which both physiological and biological changes occurred are studies by Dammann, Shappell, Bartell, & Schoenfuss (2011), Garcia-Reyero, et al. (2011), and Hyndman, Biales, Bartell, and Schoenfuss (2010). The authors observed the effects of EACs on vitellogenin biosynthesis in male fish and the decline in nest holding by male fathead minnows. All three studies observed an increase in plasma vitellogenin concentrations in estrogen exposed male fish (Dammann, Shappell, Bartell, & Schoenfuss, 2011; Garcia-Reyero, et al., 2011; Hyndman, Biales, Bartell, & Schoenfuss, 2010). However, the results for nest holding varied among the studies. In studies by Dammann, Shappell, Bartell, and Schoenfuss (2011) and Garcia-Reyero et al. (2011), the authors observed that the control minnows were more aggressive in their nest defense than the exposed minnows. In contrast, Hyndman, Biales, Bartell, and Schoenfuss (2010) observed mixed results for nest defense among experiments. In the first experiment the authors observed that control larvae out-competed steady rate exposed larvae, while in second experiment the same treatment of steady rate exposed larvae out-competed the control larvae.

The above described studies indicate that behavioral responses are vulnerable to the effects of EAC exposures. These behavioral responses consist of intricate patterns that result from the recognition of various environmental stimuli and are sensitive to the effects of exposure to aquatic pollutants (Sloman & McNeil, 2012). Given the sensitivity of behavioral responses, changes as a result of EAC exposure may occur at low concentrations, before physiological alterations are observable. The resultant behavior modifications can have ecological consequences, and may ultimately be detrimental to the survival of an organism and

sustainability of the population. Some examples of behavioral changes as a result of pollutant exposures include social interactions among conspecifics, predator avoidance, feeding efficiency, and mating (Sloman & McNeil, 2012).

An important factor in the survival of fathead minnows to reproductive maturity is their ability to avoid predators (Batty & Domenici, 2000). Foraging movements by predators create stimuli that are perceived by larvae of prey species. With a danger stimulus received, there are several known evasive strategies employed by larval fishes: either they move to shelter, reduce activity so they are less conspicuous, form tightly organized schools to reduce predator effectiveness, or swim rapidly and randomly in order to avoid/confuse the predator. The swimming rapidly and randomly response is conducted through short coordinated bursts of the tail called fast starts (Liu & Fetcho, 1999). Among the different fast-start strategies, the C-start is a well-known and studied behavior (Eaton, Lee, & Foreman, 2001; McGee, et al., 2009; Painter, et al., 2009; Temple & Johnston, 1997). This reflex behavior begins with a latency period during which the fathead minnow processes the stimulus from the predator, which is followed by a sharp bending of the minnow into a C-shape, and completed with a rapid swimming movement away from the predator stimulus (Domenici & Batty, 1997). If this behavior is delayed, the predator avoidance performance will suffer, possibly reducing the survival chances of the minnow. Multiple studies have demonstrated that effluent composites can impact the predator avoidance performance of fathead minnows (McGee, et al., 2009; Painter, et al., 2009). The physiological response of the C-start behavior, as a result of the predator stimulus, is controlled by an integrated sensory-motor axis. In the hindbrain, reticulospinal neurons MiD2 cm and MiD3 cm, assist specialized neurons, Mauther cells, to activate a musculo-skeletal response to a

perceived predator stimulus. The musculo-skeletal response or stimulation of the muscle fibers in the lateral musculature, occurs as a result of activation of Mauther cells along with MiD2 cm and MiD3 cm neurons, which excite motor neurons and interneurons (Liu & Fetcho, 1999).

In addition to predator avoidance, feeding performance is also an essential larval behavior needed for the survival of the organism. Foraging and consuming food sources found in the surrounding environment becomes a necessity after the larval yolk is fully consumed. The ability of the larval minnows to forage and capture prey is a determinant performance needed for their growth and sustainability. In experiments conducted by Grippo and Heath (2003), feeding performance was compared in fish exposed to different concentrations of methylmercury. The performance parameters that were tested included foraging efficiency, capture speed, and the minnow's ability to acquire and remember the characteristics of the habitat (Grippo & Heath, 2003). Although there were no effects on the fathead minnow's ability to acquire and remember habitat information (Grippo & Heath, 2003), the exposed minnows displayed deficits in foraging efficiency, as well as capture speed, when compared to control fathead minnows. Another study demonstrating the importance of feeding performance was conducted by Zhou, Scali, Weis, and Weis (1996). The authors observed that the exposure of *Fundulus heteroclitus* to methylmercury during the embryonic stage transiently lessened their ability to capture prey. The authors believed that the transient effects of methylmercury either (1) limited neurochemical processes, or (2) altered neurological development (which was recovered after cessation of exposure) (Zhou, Scali, Weis, & Weis, 1996). In yet another study examining the effects of methylmercury at concentrations much higher than currently found in the environment, the authors observed that feeding performance, alone and competitively against control grayling, *Thymallus thymallus*,

was reduced 3-years post-exposure (Fjeld, Haugen, & Vollestad, 1998). This study observed the longevity of behavioral modification following a brief exposure during embryonic development. In a more recent experiment conducted by Brodin, Fick, Josson, and Klaminder (2013), juvenile perch were exposed to different concentrations of the pharmaceutical oxazepam in order to test its effects on feeding performance. Results suggested that even perch exposed at low concentrations initiated feeding of first zooplankton and completed the food source of 20 zooplankton faster than the control group of perch that were unexposed (Brodin, Fick, Josson, & Klaminder, 2013). This finding raises the specter that increased feeding rates may cause the fish to deplete their food source more rapidly with catastrophic consequences to the ecosystem. Along with an increased feeding rate, the perch's activity and boldness were also found to have increased (Brodin, Fick, Josson, & Klaminder, 2013). This may increase the perch's predation risk, which could compromise their survival.

The fathead minnow, *Pimephales promelas*, is commonly used as a model to test the effects of pharmaceuticals and estrogenic compounds commonly found in wastewater effluent. One of the reasons for the popularity of the fathead minnow as a laboratory model organism is its large geographic range, which includes most of North America. This illustrates that the species is able to survive in a wide variety of environments. Another for reason the fathead minnow's popularity as an acceptable laboratory model species is its ability to quickly reach sexual maturity and begin spawning often by the end of the first summer after hatching (Held & Peterka, 1974). After reaching sexual maturity, fathead minnows are capable of producing large quantities of offspring over multiple spawns, usually separated by 4-6 days. Once the eggs are fertilized, embryos develop and hatch roughly five days post-fertilization. The larvae grow and

develop by consuming an omnivorous diet (Held & Peterka, 1974; Price, Tonn, & Paszkoski, 1991), varying from season to season. Their diet consists mainly of zooplankton, macroinvertebrates, and detritus (Held & Peterka, 1974; Price, Tonn, & Paszkoski, 1991; Zimmer, Herwig, & Laurich, 2006). As a result of the popularity of the fathead minnow as a laboratory model organism, well-developed standardized rearing and fish maintenance protocols have been established (Denny, 1987)

Study Introduction

While a majority of past studies have examined the physiological effects of pharmaceuticals and EACs, I intended to observe the biological effects through behavioral observations. Behavioral modifications may occur at much lower concentrations of exposure, before any physiological alterations may be observed. By conducting short-term exposures starting with fertilized fathead minnow eggs I will be able to assess the adverse effects of different pharmaceuticals and EACs during the important growth and developmental stage of the minnow's lifecycle. Specifically, I plan to analyze two important traits, larvae predator avoidance performance and feeding performance, that are essential to reproductive fitness. Consequently, this study will test the following two hypotheses.

First, pharmaceutical and E1 exposure will reduce predator avoidance performance in fathead minnows. In previous studies with fathead minnows exposed to antidepressants (Painter, et al., 2009) or multiple estrogens (McGee, et al., 2009), the authors observed a reduction in predator avoidance performance. In a study conducted by Zhou, Scali, Weis, and Weis (1996), the authors tested the predator avoidance performance of mummichog larvae exposed to

methylmercury. When ten mummichog larvae were placed in a tank for 24 hours with three natural predators, grass shrimp, the survival was reduced in correlation with increased methylmercury exposure (Zhou, Scali, Weis, & Weis, 1996). Thus the ability to avoid predators became more impeded as the exposure concentration increased.

Second, pharmaceutical and E1 exposure will reduce feeding performance in fathead minnows. The study conducted by Grippo and Heath (2003) corroborates this hypothesis. The authors demonstrated that fathead minnows exposed to methylmercury experienced decreased foraging abilities. The authors attributed this decreased foraging efficiency to either hunger suppression, increased amount of pause time, or impairment of the senses (Grippo & Heath, 2003). Any of these could result in a decreased feeding rate of fathead minnows as they would cause fathead minnows to become less efficient feeders. In addition, studies have demonstrated that crucian carp, *Carassius auratus,* when exposed to sertraline, had diminished foraging abilities (Xie, et al., 2015). The authors observed that the crucian carp exposed to sertraline needed more time to capture an equal quantity of midge larvae when compared to control fish. This demonstrates how pharmaceuticals with different modes of action may produce similar results. The current study will examine if pharmaceuticals or estrogens, compounds with varying modes of action, diminish either predator avoidance performance or feeding performance.

Chapter II

THE EFFECTS OF PHARMACEUTICAL EXPOSURE ON PREDATOR AVOIDANCE PERFORMANCE AND FEEDING EFFICIENCY IN LARVAL FATHEAD MINNOWS

Introduction

Pharmaceuticals are widely used and have been detected in treated wastewater effluent that is discharged into waterways (Koplin, et al., 2002; Richardson, 2012; Schultz, et al., 2011). The persistence of pharmaceuticals in wastewater is due to their property of being metabolically stable, making them less likely to be degraded during the wastewater treatment processes (Dietrich, Webb, & Petry, 2002; Glassmeyer, et al., 2005). In addition, wastewater treatment plants are not engineered to remove pharmaceuticals, allowing them to persist and remain biologically active after treatment and release into waters (Ying, Kookana, & Koplin, 2009). A wide range of pharmaceutical compounds are being released, causing environmental concern regarding their potential toxicity and unknown effects on non-target organisms.

Reproduction fitness is imperative to the survival of a species. Non-reproductive traits, such as behavioral responses, play a crucial role in survival to reproduction (Batty & Domenici, 2000). These behavioral responses are sensitive to the exposure of aquatic pollutants (Sloman & McNeil, 2012) and behavioral modifications resulting from exposure could be detrimental to the survival of an organism. Some examples of behaviors that may be altered in pollution exposed fish include social interactions, predator avoidance, feeding, and mating (Sloman & McNeil, 2012). In order to reach sexual maturity, defined as the biological capability to mate, fish must

successfully avoid predators and forage efficiently to obtain adequate food to grow and remain healthy.

An important factor in the survival of fathead minnows is their ability to avoid predators (Batty & Domenici, 2000). Foraging movements by predators create stimuli that are perceived by larvae of prey species. With a danger stimulus received, there are several known evasive strategies employed by larval fishes: either they move to shelter, reduce activity so they are less conspicuous, form tightly organized schools to reduce predator effectiveness, or swim rapidly and randomly in order to avoid/confuse the predator. Rapid and random swimming is achieved through short coordinated bursts called fast starts (Liu & Fetcho, 1999). Among the different fast-start strategies, the C-start is a well-studied behavior (Eaton, Lee, & Foreman, 2001; McGee, et al., 2009; Painter, et al., 2009; Temple & Johnston, 1997). This reflex behavior begins with a latency period, during which the fish processes the stimulus from the predator, which is followed by a sharp bending of the fish into a C-shape, and completed with a rapid swimming movement away from the predator stimulus (Domenici & Batty, 1997). If this behavior is delayed, the predator avoidance performance will suffer, possibly reducing the survival chances of the minnow. Multiple studies have demonstrated that effluent composites can impair predator avoidance performance in fathead minnows (McGee, et al., 2009; Painter, et al., 2009). The physiological response of the C-start behavior, as a result of the predator stimulus, is controlled by an integrated sensory-motor axis. In the hindbrain, reticulospinal neurons, MiD2 cm and MiD3 cm assist specialized neurons, Mauther cells, to activate a musculo-skeletal response to a perceived predator stimulus. The musculo-skeletal response or stimulation of the muscle fibers in

the lateral musculature, occurs as a result of activation of Mauther cells along with MiD2 cm and MiD3 cm neurons, which excite motor neurons and interneurons (Liu & Fetcho, 1999).

Another important factor for survival is the feeding performance of larval fish. Foraging ability will affect the growth and development of the animal (Xie, et al., 2015). In experiments conducted by Grippo and Heath (2003), feeding performance was evaluated relative to exposure to different concentrations of methylmercury. The authors tested foraging efficiency, capture speed, and the minnow's ability to acquire and remember the characteristics of its habitat (Grippo & Heath, 2003). The exposed minnows displayed deficits in foraging efficiency, as well as capture speed, when compared to the control fathead minnows, although, there were no effects on the fathead minnow's ability to acquire and remember habitat information (Grippo & Heath, 2003). Zhou, Scali, Weis, and Weis (1996) found that the exposure of *Fundulus heteroclitus* to methylmercury during the embryonic stage lessened their ability to capture prey. The authors believed that the transient effects of methylmercury either (1) limited neurochemical processes, or (2) altered neurological development (which was recovered by the minnows) (Zhou, Scali, Weis, & Weis, 1996). Another study done by Nassef et al. (2010) observed medaka, *Oryzias latipes*, exposed to carbamazepine or diclofenac in acute exposure resulted in altered feeding performance. Besides feeding performance, swimming speed was observed to be altered by carbamezapine, but not diclofenac. This result indicated that fish are affected by pharmaceuticals through different mechanisms (Nassef, et al., 2010). A third study conducted by Bodin, Fick, Josson, and Klaminder (2013) observed that wild European perch, *Perca fluviaatilis,* exposed to oxazepam, modified behavior and foraging rate. The exposed wild European perch exhibited

increased activity and an increased feeding rate (Brodin, Fick, Josson, & Klaminder, 2013). Further research is needed to see if different chemicals produce a similar behavioral response.

As many pharmaceuticals remain metabolically active after being processed through wastewater treatment plants, different classes of pharmaceuticals reach aquatic ecosystems. These pharmaceuticals have different mechanisms of action and may impact the native species in different ways. Some commonly observed classes of pharmaceuticals in wastewater include muscle relaxants, statins, antibiotics, benzodiazepines, and non-steroidal anti-inflammatories (NSAIDs).

An interesting pharmaceutical pollutant is the muscle relaxant methocarbamol (3-o-methoxyphenoxy-2-hydroxypropyl-carbamate), also known by its brand name Robaxin. The exact mode of action for this pharmaceutical is unknown. However, methocarbamol is viewed as a central nervous system depressant that results in relaxing skeletal muscle (Sannerud, Ator, & Griffiths, 1991). Methocarbamol accomplishes this effect by inhibiting polysynaptic pathways in the spinal cord (Sannerud, Ator, & Griffiths, 1991; Truitt & Maxwell Little , 1958). Interestingly, there are no published concentration data for methocarbamol in effluents. However, the U.S. Geological Survey had observed to be present at concentrations up to 2,310 ng L^{-1} (unpublished data).

Statins are used in humans to treat high cholesterol and triglyceride levels. Statins inhibit 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR) from catalyzing the conversion of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate in the cholesterol biosynthesis pathway (Holdgate, Ward, & Mctaggart, 2003). The reduced cholesterol levels cause up-

regulation of low-density-lipoprotein (LDL) receptors, which bind available LDL in plasma (Brown & Goldstein, 1986). A commonly prescribed statin is rosuvastatin, also known as Crestor, which has been observed to be present at concentrations up to 552 ng L^{-1} in effluent waters in Canada (Lee, Peart, Svoboda, & Backus, 2009), and 979 ng L^{-1} in Europe (Loos , et al., 2013).

Antibiotics, including sulfonamides, are common aquatic pollutants. Sulfonamides are antagonists for dihydropteroate synthase, inhibiting the conversion of Mg-6-hydroxymethyl-7,8-dihydropterin diphosphate and para-aminobenzioc acid to Mg-pyrophosphate and 7,8-dihydropteroate (Valderas, Andi, Barrow, & Cook, 2008). Thus, sulfonamides inhibit bacteria's folate biosynthesis pathway. A commonly used sulfonamide is sulfamethoxazole, which is often found in effluent waters. It has been observed to be present at concentrations up to 1.9 µg L^{-1} in streams sampled across the United States (Koplin, et al., 2002).

Benzodiazepines are mainly used to treat anxiety disorders, but can also be used to treat sleep disorders and epilepsy (Baily, Ward, & Musa, 1994). They increase the binding potential of gamma amino butyric acid (GABA), which is classified as an inhibitory neurotransmitter (Baily, Ward, & Musa, 1994). A common benzodiazepine is temazepam, also known as Restoril, which is a metabolite of diazepam, and has been observed to be present up to 1016 ng L^{-1} in effluent waters in the Netherlands (van der Aa, et al., 2013).

Another popular class of pharmaceuticals often observed as aquatic pollutants are NSAIDs. These pharmaceuticals inhibit cyclooxygenase-1(COX-1) and -2 (COX-2), preventing prostaglandin biosynthesis (Vane & Botting, 1998). A very popular NSAID is diclofenac, also

known as Voltaren, which is used in both human and veterinary practice. It is also well known for its adverse ecological effects, causing the populations of the *Gyps* vultures in India that were scavenging on the carcasses of livestock treated with diclofenac, to decline to near extinction in the 1990's (Prakash, et al., 2003). In aquatic ecosystems diclofenac has been observed to be present at concentrations up to 15 µg L^{-1} (Jux , Baginiski, Hans-Gunter, Kronke, & Seng, 2002). When exposed to 5 µg L^{-1} diclofenac, rainbow trout, *Oncohynchus mykiss*, developed renal lesions and morphologic changes to gill tissue (Schwaiger, Ferling, Mallow, Wintermayr, & Negele, 2004). The authors also found bioaccumulation of diclofenac in the liver, kidney, gills, and muscle tissue (Schwaiger, Ferling, Mallow, Wintermayr, & Negele, 2004). In Japanese medaka, *Oryzias latipes*, foraging efficiency was decreased when fish were exposed to 0.17 mg L^{-1} of diclofenac for 9 days (Nassef, et al., 2010).

In the present study, we exposed fathead minnow larvae to either diclofenac, methocarbamol, rosuvastatin, sulfamethoxazole or temazepam and tested two important non-reproductive traits that are significant to reproductive fitness. Deficiencies in either predator avoidance performance or feeding performance may result in an organism not reaching maturity to reproduce. The objective of this study was, therefore, to test two hypotheses. First, if larvae were exposed to either diclofenac, methocarbamol, rosuvastatin, sulfamethoxazole or temazepam, they would exhibit a diminished predator avoidance response. Secondly, exposure to the pharmaceuticals would reduce the larvae's feeding performance.

Table 1: Mode of action for experiment pharmaceuticals.

Drug	**Mode of Action**
Methocarbamol	Unknown
Rosuvastatin	HMG-CoA reductase competitive inhibitor
Sulfamethoxazole	Antagonist of dihydropteroate synthase
Temazepam	Increase binding of gamma-aminobutyric acid (GABA) at receptor
Diclofenac	Inhibits cyclooxygenase (COX)

Methodology

Fish

Six month old male and female fathead minnows, *Pimephales promelas*, were obtained from a laboratory fish culture (Environmental Consulting and Testing, Superior, WI). These fish (F1 generation) were split into four breeding aquaria groups of five males and five females each. Each breeding group was placed in an 80 L aquaria containing multiple spawning tiles and allowed to breed. Eggs were collected daily, placed into two separate aquaria, allowed to hatch, and then grown into adults. After six months, the second generation of fathead minnows (F2 generation) were used as breeding pairs for this experiment. Sixty 8 L aquaria were set up with one male and two females each. The aquaria each contained an air stone and two spawning tiles, and were supplied with flowing well water at 21°C. The adults were fed *ad libitum* with frozen brine shrimp and blood worms (Brine Shrimp Direct, Salt Lake City, UT) twice daily. Eggs collected from these breeding pairs were used for this experiment.

Test Chemicals

The pharmaceuticals stock solutions for the exposure experiments were prepared by the US Geological Survey (Denver, CO) using ethanol as a solvent. Each pharmaceutical stock solution was diluted with well water to experimental concentrations in 50 1 L Teflon-coated containers, which were filled to approximately 0.8 L and frozen until use in the current study. The containers were filled with either an ethanol solvent control, methocarbamol (4,800 ng L^{-1}), rosuvastatin (3,200 ng L^{-1}), sulfamethoxazole (2,200 ng L^{-1}), temazepam (1,600 ng L^{-1}) or

diclofenac (1,600 ng L^{-1}). The US Geological Survey (Denver, CO) confirmed the aqueous concentrations through analytical chemistry (LC/MS/MS).

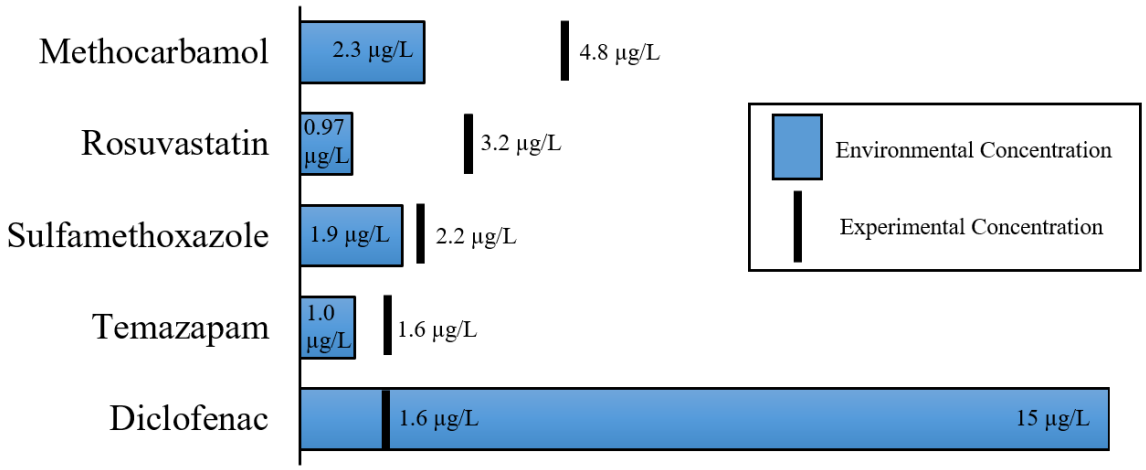

Figure 1: Experimental concentrations and max environmental concentrations for methocarbamol, rosuvastatin, sulfamethoxazole, temazepam and diclofenac.

Exposure

Once eggs were deposited on a breeding tile by the adult fathead minnows, the tile was removed and placed in 0.9% hydrogen peroxide for ten minutes to disinfect and remove any harmful contaminants that could prevent larvae from developing properly (Marking, Rach, & Schreier, 1994). Next, the tile was placed in a one liter beaker containing conditioned well water (well-water that was aerated heavily for the prior 24 hrs) and an air stone with bubbles slowly rolling over the eggs. Once the eye spots appeared on the larva, an indication of successful fertilization and early development, the fertilized eggs were rolled off the spawning tile with a spatula into a beaker with conditioned well water. Twenty eggs each were transferred using a three milliliter eye dropper into a homemade butterfly mesh basket within an exposure aquaria with an air stone slowly bubbling underneath. Each exposure aquarium contained one basket and was treated with one of the pharmaceuticals. The compounds tested included diclofenac, methocarbamol, rosuvastatin, sulfamethoxazole, and temazepam. In addition, a 500 $\mu g\ L^{-1}$ ethanol solvent control, matching the ethanol used to solubilize the other contaminants, was established. The air stone and basket were removed once the larvae hatched. Hatched brine shrimp (Brine Shrimp Direct, Salt Lake City, UT), 1 mL, were placed in each aquaria twice daily to allow the larvae to feed *ad libitum* (Painter, et al., 2009; McGee, et al., 2009). A 50% static renewal water exchange was completed for each aquaria daily during the twenty-one days of exposure (Rearick, et al., 2014). This was accomplished by removing half the water from the aquaria using an air-line hose connected to a syringe with butterfly mesh placed over one end,

preventing larvae from suction and harm, and replacing it with the corresponding treatment water. The exposure was replicated nine times. Each replicate using a different clutch of offspring.

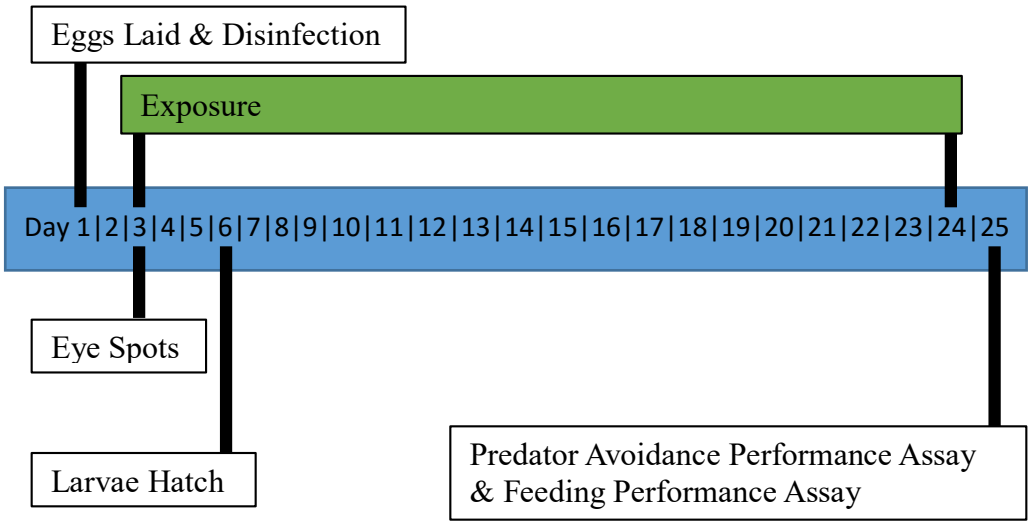

Figure 2: Experimental timeline for pharmaceutical exposure. Total experiment timeframe is displayed in blue box. Text boxes display events.

Predator Avoidance Performance Assay

The purpose of the predator avoidance assay was to test the effects of the different pharmaceuticals on predator avoidance performance. To test predator avoidance performance, a trigger-activation system was used following procedures by McGee et al., (2009) with some alterations. A five centimeter petri dish containing a larval minnow in 10 mL of conditioned well water was placed on a 1 mm grid, allowing the larva's movements to be quantified. Under the grid, there was an electronic chip that when activated, emitted vibrations, simulating an approaching predator. A red LED light (shielded in such a way that the larva was not able to see the light) was also connected to the electronic chip and activated simultaneously with the electronic chip. The LED light was used to indicate time zero during the analysis of the recording sequences. A high speed digital video camera (Motion Scope M1 by AOC Technologies) capable of capturing 1000 frames per second was placed 50 cm above the grid in order to capture the entire filming area. The night before testing, larvae were transported to the testing room to acclimate to ambient conditions. The water used in the petri dish was conditioned well water, kept at room temperature. Twenty minutes prior to testing, the larvae were fed *ad libitum* with brine shrimp. One performance recording was conducted for three larval minnows in each treatment. The test started after the fish acclimated to the petri dish and swam into the center portion of the grid, marked with a square, and paused. Once inside the center portion, the electronic chip was activated by pressing a trigger to elicit the predator avoidance response. The high-speed film recordings were saved as .AVI files and examined with the Image J program for Windows 7. The anterior tip of the head and posterior tip of the tail for each larva were marked, along with two dots on the one millimeter grid. The data was transferred to Microsoft Excel and

used to calculate time of initiation (latency period), escape velocity (body lengths/ms during the first forty milliseconds after initiation), the total escape response (body lengths/(latency period in ms + 40 ms)), and the angle of predator avoidance escape (angle of bend after initiation of predator avoidance response) (Figure 3). The velocity was converted to body lengths per millisecond to remove the effect of body size on predator avoidance performance. In order for the videos to be considered for analysis, each recording needed (1) to have a latency response of more than five milliseconds, (2) have their first response after predator stimulus be an escape turn (Liu & Fetcho, 1999), and (3) stay in the field of view for the length of the recording. The exclusion of some recordings that did not meet these criteria resulted in some differences among sample sizes between treatments.

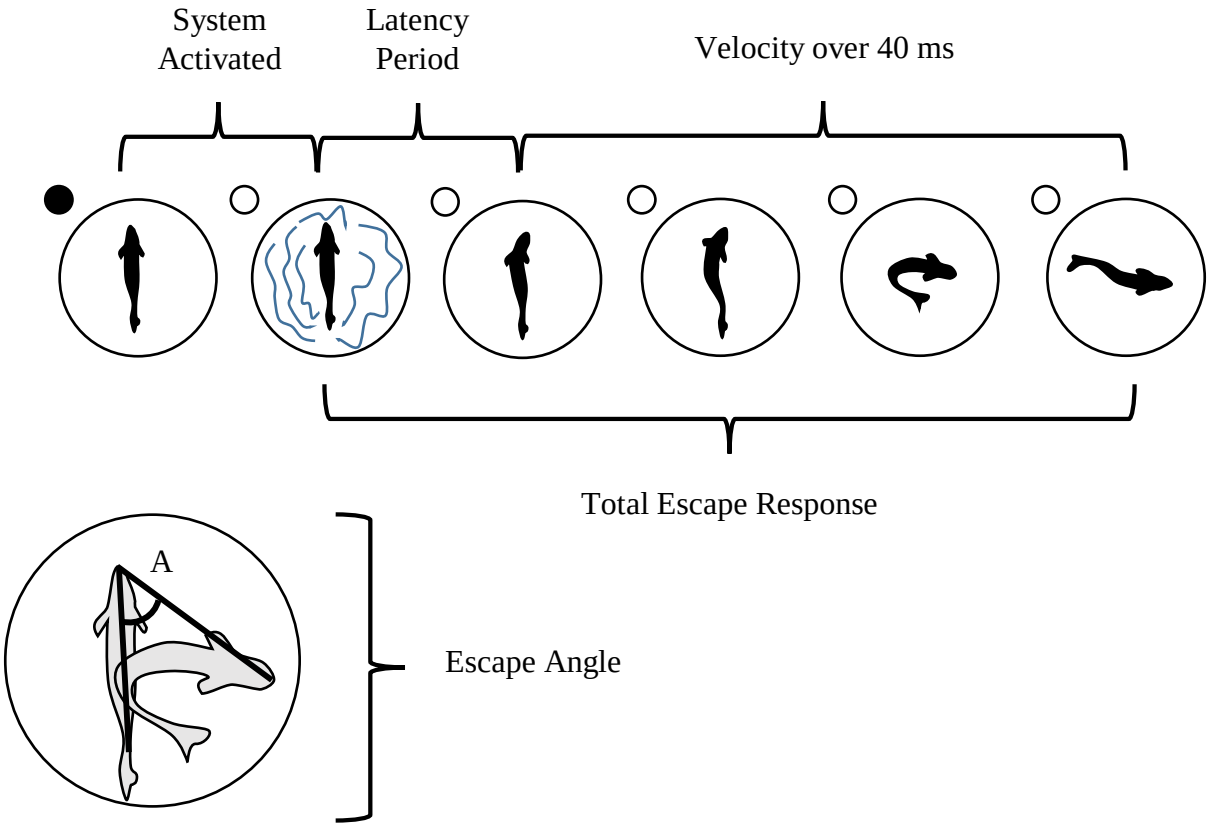

Figure 3: Schematic representation of predator avoidance behavior with measured performance endpoints. When the system is activated the vibrational stimulus begins and the LED light is turned on, indicating time zero of test. The time in milliseconds from the vibrational stimulus to the first head movement was defined as the latency period. The subsequent 40 ms after first head movement is used to determine the escape velocity in body length per milliseconds. Both Latency period and escape velocity were combined to formulate the total escape response. The escape angle was calculated by subtracting the angle of furthest extent, A, from 180°.

Feeding Performance Assay

In this experiment, the larvae from each treatment were assessed for feeding performance which was defined as a larva's capability to consume as many brine shrimp as possible within a one minute interval (Figure 4). Six larvae, two larvae per well, from each treatment were placed into six well plates (n=3 per treatment). The diameter of each well was 35 mm and contained 10 mL of conditioned well water. The larvae remained in the wells for at least 12 hrs before the testing took place. This allowed sufficient time of deprivation to empty the stomachs of the larvae without compromising their health. Before each test, the larvae were given 1 min to acclimate, since moving the wells could cause stress to the larvae. During the test, the larvae were given 1 min after 25 to 35 brine shrimp were placed in the well to eat as many brine shrimp as possible. After one minute the larvae were euthanized with a sodium bicarbonate MS-222 solution (230 g MS-222, 115 g sodium bicarbonate per 1 L of well water). The remaining brine shrimp were then counted to determine how many brine shrimp the larvae ate.

Statistical Analyses

All analysis of the data was conducted in the statistical software package Prism version 4.01 (GraphPad Software Inc.). First the data for the three trials in the predator avoidance performance assay and the feeding performance assay were averaged so that n=1 for each replicate. Next, the escape angle and the percentage of brine shrimp eaten were arcsine transformed. Then for each treatment, each endpoint was assessed by an unpaired T-test with equal standard deviations against controls. A p-value of less than 0.05 was considered significant.

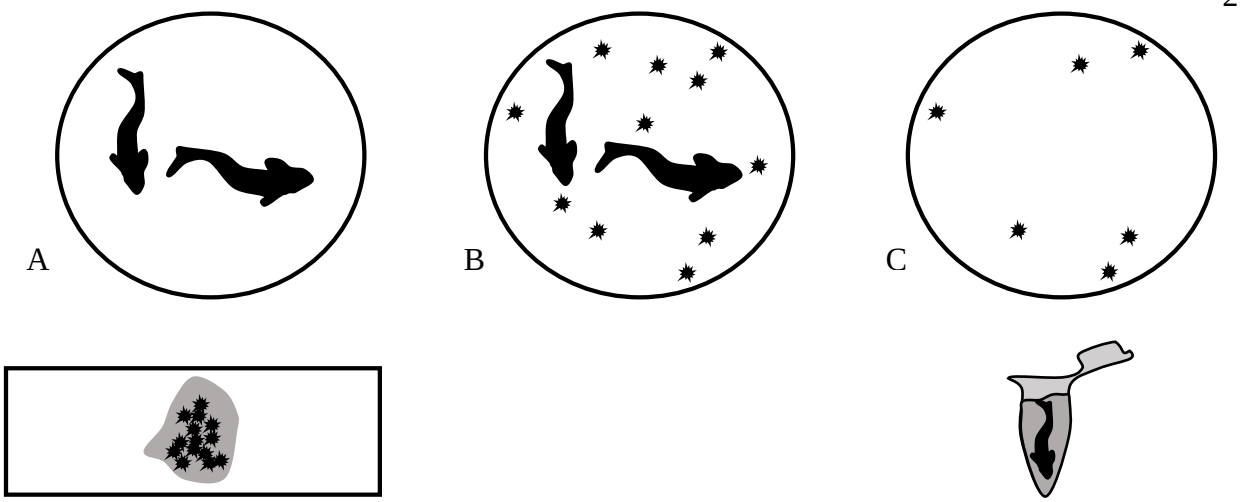

Figure 4: Schematic representation of the feeding performance assay. A: Fish were allowed to acclimate and 25-35 brine shrimp were counted on a slide B: Brine shrimp were rinsed with conditioned well water into well, time zero, initiating feeding performance test. C: After 1 min larvae were euthanized with a MS-222 solution. Larvae were removed and placed in RNA-later. The remaining brine shrimp were then counted.

Results

Larval Hatch and Survival

Looking at the hatching success, the percentage of larvae that hatch from fertilized embryos, the mean hatch success was 81.67% for control larvae, 87.22% for methocarbamol (4,800 ng L^{-1}) exposed larvae, 87.22% for rosuvastatin (3,200 ng L^{-1}) exposed larvae, 87.22% for sulfamethoxazole (2,200 ng L^{-1}) exposed larvae, 88.89% for temazepam (1,600 ng L^{-1}) exposed larvae, and 88.89% for diclofenac (1,600 ng L^{-1}) exposed larvae. From hatching to testing the mean survival percentage was 97.36 for control larvae, 96.19% for methocarbamol (4,800 ng L^{-1}) exposed larvae, 94.91% for rosuvastatin (3,200 ng L^{-1}) exposed larvae, 95.74% for sulfamethoxazole (2,200 ng L^{-1}) exposed larvae, 94.93% for temazepam (1,600 ng L^{-1}) exposed larvae, and 95.82% for diclofenac (1,600 ng L^{-1}) exposed larvae.

Larval Predator Avoidance Performance

When tested 21 days post embryonic eyespot development, larvae exposed to either rosuvastatin (3,200 ng L^{-1}) or diclofenac (1,600 ng L^{-1}) had longer predator avoidance latency periods compared to the controls ($p=0.03$ and $p=0.027$) (Figure 5B). The mean of the latency periods for the rosuvastatin (3,200 ng L^{-1}) and diclofenac (1,600 ng L^{-1}) exposed larvae were roughly 135% greater than the latency period of the control larvae. The increased C-start latency periods for the rosuvastatin (3,200 ng L^{-1}) and diclofenac (1,600 ng L^{-1}) exposed larvae did not carry over into either a slower escape velocity, variable escape angle, or slower total escape response (Figure 5). Exposure to methocarbamol (4,800 ng L^{-1}), sulfamethoxazole (2,200 ng L^{-1}) or temazepam (1,600 ng L^{-1}) did not result in significant differences in latency period, escape

angle, escape velocity or total escape response (Figure 5). All larvae, independent of treatment, grew to a similar size.

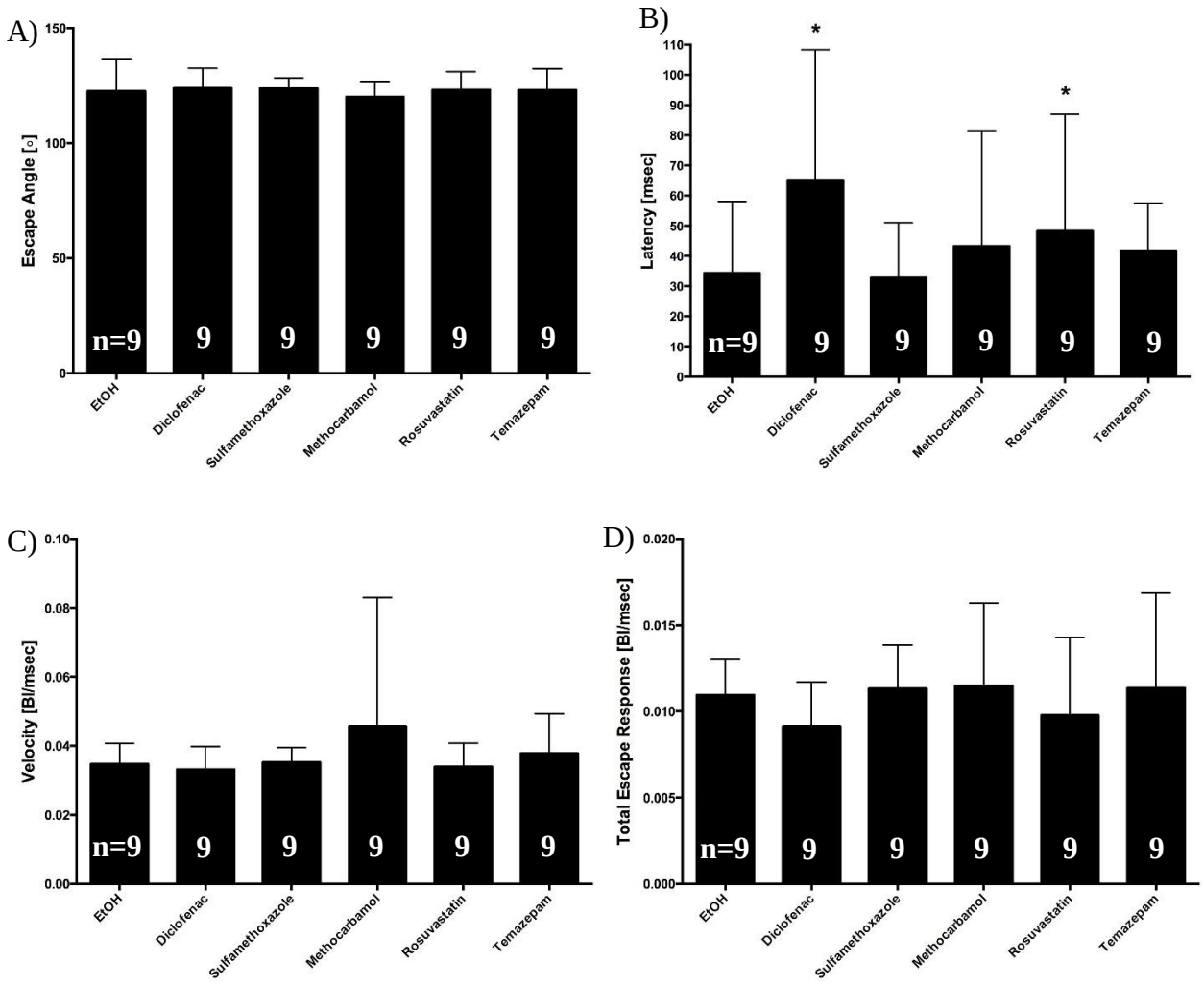

Figure 5: Effects of 21-day exposure to single pharmaceuticals on predator avoidance performance in larval fathead minnows. (A) mean escape angle, 180° minus the angle measured for farthest extent of bend; (B) mean of latency period, time in msec from initiation of stimulus, indicated by light turning on, to first head movement of predator avoidance performance; (C) mean escape velocity for 40 ms measured in body length per ms; (D) mean total escape response from stimulus until 40 ms post first head movement. These values combine latency period with escape velocity of predator avoidance response. Statistical significance was assigned when $p<0.05$ and indicated by an asterisk (*).

Larval Feeding Performance

When tested 21 days post embryonic eyespot visualization, larvae exposed to rosuvastatin (3,200 ng L^{-1}), diclofenac (1,600 ng L^{-1}), methocarbamol (4,800 ng L^{-1}), sulfamethoxazole (2,200 ng L^{-1}) and temazepam (1,600 ng L^{-1}) foraged at similar rates. There were no significant differences for percentage of brine shrimp eaten (Figure 6).

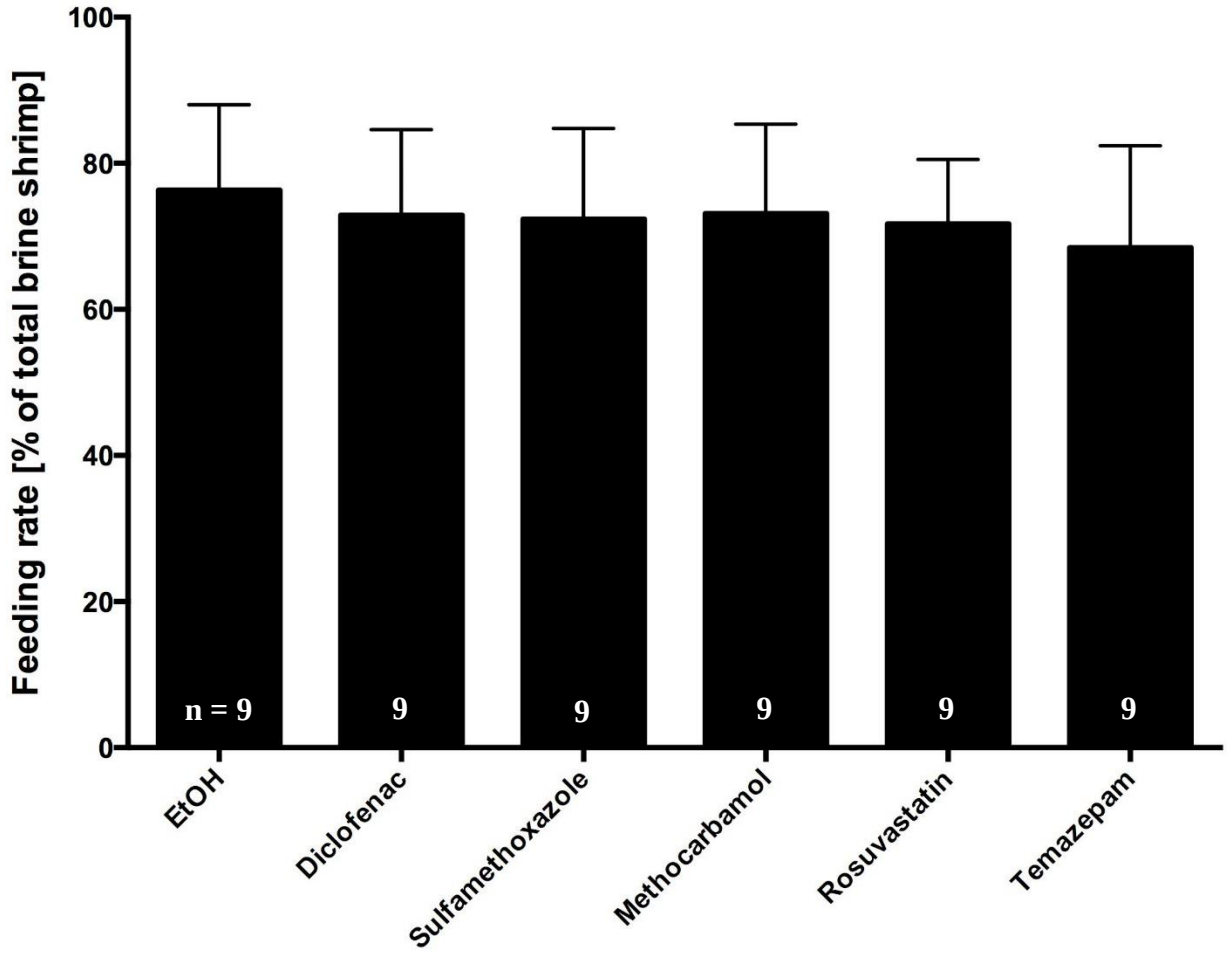

Figure 6: Effects of 21-day exposure to single pharmaceuticals on feeding performance in larval fathead minnows. Feed rate was determined by percentage of brine shrimp consumed in one minute. Statistical significance was assigned when $p<0.05$ and indicated by an asterisk (*).

Chapter III

THE EFFECTS OF ESTRONE EXPOSURE ON PREDATOR AVOIDANCE PERFORMANCE AND FEEDING EFFICIENCY IN LARVAL FATHEAD MINNOWS

Introduction

Steroidal and hormonal compounds, also known as endocrine active compounds (EACs), have been found in treated wastewater effluents discharged into aquatic environments (Desbrow, Routledge, Brighty, Sumpter, & Waldock, 1998; Koplin, et al., 2002). Among EACs are estrogens, hormones found in both humans and aquatic wildlife (Colborn, vom Saal, & Soto, 1993). Some common estrogens that are found in the environment are estrone (E1), 17β-estradiol (E2), 17α-ethynlestradiol (EE2) and estriol (E3). These estrogens as aquatic pollutants have been observed to cause deleterious consequences to exposed fishes (Kidd, et al., 2007).

Reproductive fitness has been observed to decrease with exposure to EE2 in a whole-lake exposure (Kidd, et al., 2007). Near depletion of the native populations resulted due to the feminization of males and abnormal oogenesis in female fish (Kidd, et al., 2007). Reproductive fitness is crucial to the survival of a species and is affected by non-reproductive traits such as behavioral responses, which impact survival to reproductive maturity (Batty & Domenici, 2000). These behavioral responses are sensitive to the exposure to aquatic pollutants (Sloman & McNeil, 2012), but were not part of the Kidd et al. (2007) study. Behavioral modifications resulting from exposure could be detrimental to the survival of an organism. Some examples of behavioral changes that may be altered by EACs exposure are: social interactions, predator

avoidance performance, feeding efficiency, and mating (Sloman & McNeil, 2012). In order to reach sexual maturity, defined as the biological capability to mate, fish must successfully avoid predators and forage efficiently to obtain adequate food to grow and remain healthy.

An important factor in the survival of fathead minnows is their ability to avoid predators (Batty & Domenici, 2000). Foraging movements by predators create stimuli that are perceived by larvae of prey species. With a danger stimulus received, there are several responses which fathead minnows are known to exhibit: either they move to shelter, reduce activity so they are less conspicuous, form tightly organized schools to reduce predator effectiveness, or swim rapidly and randomly in order to avoid/confuse the predator. Swimming rapidly and randomly is achieved through short coordinated bursts called fast starts (Liu & Fetcho, 1999). Among the different fast-start strategies, the C-start is a well-studied behavior (Eaton, Lee, & Foreman, 2001; McGee, et al., 2009; Painter, et al., 2009; Temple & Johnston, 1997). This reflex behavior begins with a latency period when the fish is registering the stimulus from the predator, which is followed by a sharp bending of the body into a C-shape, and completed with a rapid swim movement away from the predator stimulus (Domenici & Batty, 1997). If this behavior is delayed, the predator avoidance performance will suffer, possibly reducing survival chances. Multiple studies have demonstrated that estrogenic pollutants in effluent can impact the predator avoidance performance of fathead minnows (McGee, et al., 2009). The physiological response of the C-start behavior, as the result of the predator stimulus, is controlled by an integrated sensory-motor axis. In the hindbrain reticulospinal neurons, MiD2 cm and MiD3 cm, assist specialized neurons, Mauther cells, to activate a musculo-skeletal response to a perceived predator stimulus. The musculo-skeletal response or stimulation of the muscle fibers in the lateral musculature,

occurs as a result of activation of Mauther cells along with MiD2 cm and MiD3 cm neurons, which excite motor neurons and interneurons (Liu & Fetcho, 1999).

Another important factor for survival is a minnow's feeding performance. Foraging ability will affect the growth and development of the fish (Xie, et al., 2015). In experiments conducted by Grippo and Heath (2003), feeding performance was evaluated in relation to different concentrations of methylmercury. The factors that were tested included foraging efficiency, prey capture speed, and the minnow's ability to acquire and remember the characteristics of habitat (Grippo & Heath, 2003). The exposed minnows displayed deficits in foraging efficiency, as well as capture speed, when compared to control fathead minnows. Although, there were no effects on the fathead minnow's ability to acquire and remember habitat information (Grippo & Heath, 2003). Zhou, Scali, Weis, and Weis (1996) found that the exposure of *Fundulus heteroclitus* to methylmercury during the embryonic stage lessened their ability to capture prey. The authors believed that the transient effects of methylmercury either (1) limited neurochemical processes, or (2) altered neurological development (which was recovered by the minnows) (Zhou, Scali, Weis, & Weis, 1996).

Of the common estrogens, E1 is often detected at higher concentrations than E2, EE2 and E3 in aquatic environments (Dammann, Shappell, Bartell, & Schoenfuss, 2011; Koplin, et al., 2002; Vajda, et al., 2008; Writer, et al., 2010). It has been observed to be present concentrations up to 298 ng L^{-1} in streams in the United States (Alarez, et al., 2013).

In the present study, we exposed fathead minnow larvae to E1 and tested two important non-reproductive traits that are significant to reproductive fitness. Deficiencies in either predator

avoidance performance or feeding performance may result in an organism not reaching maturity to reproduce.

Methodology

Fish

Six month old male and female fathead minnows, *Pimephales promelas*, were obtained from a laboratory fish culture (Environmental Consulting and Testing, Superior, WI). These fish (F1 generation) were split into four breeding aquaria groups of five males and five females each. Each breeding group was placed in an 80 L aquaria containing multiple spawning tiles and allowed to breed. Eggs were collected daily, placed into two separate aquaria, allowed to hatch, and then grown into adults. After six months, the second generation of fathead minnows (F2 generation) were used as breeding pairs for this experiment. Sixty 8 L aquaria were set up with one male and two females each. The aquaria each contained an air stone, two spawning tiles and were supplied with flowing well water at 21°C. The adults were fed *ad libitum* with frozen brine shrimp and blood worms (Brine Shrimp Direct, Salt Lake City, UT) twice daily. Eggs collected from these breeding pairs were used for this experiment.

Test Chemicals

Ethanol control, E1 at 25 ng L^{-1} and at 625 ng L^{-1} water were diluted from stock solutions prepared by the US Geological Survey (Denver, CO) and aqueous concentrations were confirmed by analytical methods (LC/MS/MS).

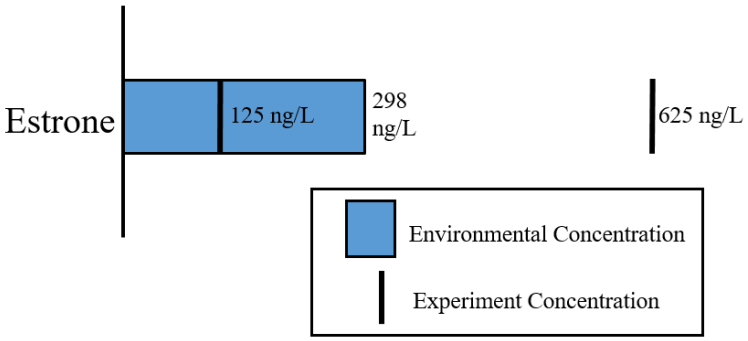

Figure 7: Experimental concentrations and max environmental concentration for estrone.

Exposure

Once eggs were deposited on a breeding tile by the adult fathead minnows, the tile was removed and placed in 0.9% hydrogen peroxide for ten minutes to disinfect and remove any harmful contaminants that could prevent larvae from developing properly (Marking, Rach, & Schreier, 1994). Next, the tile was placed in a one liter beaker containing conditioned well water (well-water that was aerated heavily for the prior 24 hrs) and an air stone with bubbles slowly rolling over the eggs. Once the eye spots appeared on the eggs, an indication of successful fertilization and early development, the fertilized eggs were rolled off the spawning tile with a spatula into a beaker with conditioned well water. Twenty eggs each were transferred using a three milliliter eye dropper into a homemade butterfly mesh basket within an exposure aquaria with an air stone slowly bubbling underneath. Each exposure aquarium contained one basket and was treated with one of the two E1 concentrations or the ethanol control. The air stone and basket were removed once the larvae hatched. Hatched brine shrimp (Brine Shrimp Direct, Salt Lake City, UT), 1 mL, were placed in each aquaria twice daily to allow the larvae to feed *ad libitum* (Painter, et al., 2009; McGee, et al., 2009). A 50% static renewal water exchange was conducted daily during the twenty-one days of exposure (Rearick, et al., 2014). This was accomplished by removing half the water from aquaria using an air-line hose connected to a syringe with butterfly mesh placed over one end, preventing larvae from suction and harm, and replacing it with corresponding treatment water. The exposure was replicated ten times. Each replicate using a different clutch of offspring.

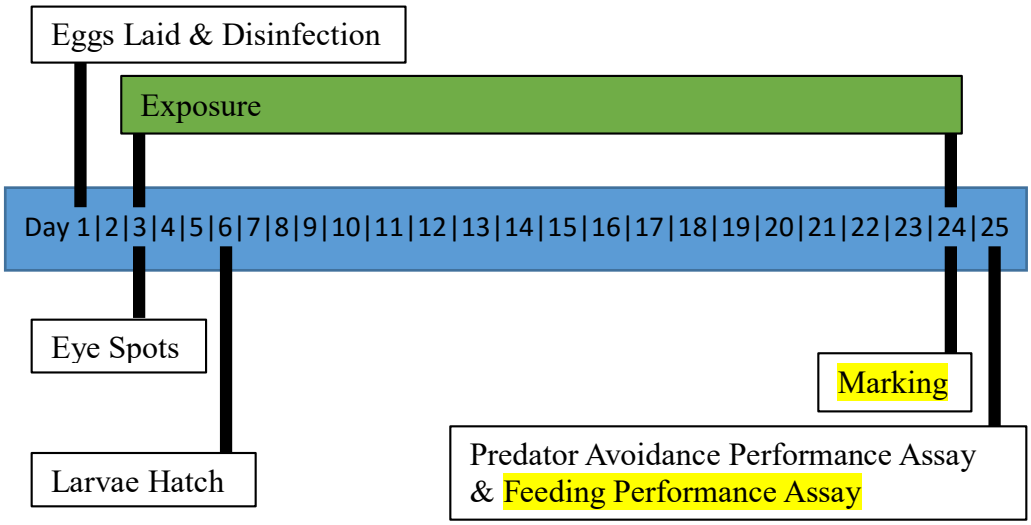

Figure 8: Experimental timeline for estrone exposure. Total experiment timeframe is displayed in blue box. Text boxes display events. Highlighted text displays differences from pharmaceutical exposure experimental timeline.

Marking

Fish were marked with a calcein dye (Western Chemical) to differentiate between control and exposed treatment larvae in the competitive feeding assay. For each replicate random number generation decided whether control or exposed larvae would be marked. The marking methodology followed the Investigational New Animal Drug (INAD 10-987) study protocol (Mohler, 2003). As specified in the protocol, all individuals that were marked weighed less than two grams. On the day before testing, the treatment larvae chosen by random number generation were placed in jars containing 100 mL of conditioned well water and 0.4 mL of 1% calcein stock solution, INAD option A 250 mg L^{-1}. The fish remained in the jars with the marking solution for six hours. The marked fish were then removed with a glass pipette and the larvae were allowed to swim out of the pipette into a jar containing 200 mL of conditioned well water. Most of the solution containing the dye marker remained in the pipette during transfer and was placed back into the marking container after the larvae exited the pipette. Any calcein marker that was accidently transferred to the new jar was removed to prevent buildup on larvae. The larvae rested overnight without food to prepare for competitive feeding assay. On the day of behavioral testing, the larvae were viewed to verify marking with the SE-Mark detector (Western Chemical).

Predator Avoidance Performance Assay

The purpose of the predator avoidance assay was to test the effects of the different pharmaceuticals on predator avoidance performance. To test predator avoidance performance, a trigger-activation system was used following procedures by McGee et al., (2009) with some

alterations. A five centimeter petri dish containing a larval minnow in 10 mL of conditioned well water was placed on a 1 mm grid, allowing the larva's movements to be quantified. Under the grid, there was an electronic chip that when activated, emitted vibrations, simulating an approaching predator. A red LED light (shielded in such a way that the larva was not able to see the light) was also connected to the electronic chip and activated simultaneously with the electronic chip. The LED light was used to indicate time zero during the analysis of the recording sequences. A high speed digital video camera (Motion Scope M1 by AOC Technologies) capable of capturing 1000 frames per second was placed 50 cm above the grid in order to capture the entire filming area. The night before testing, larvae were transported to the testing room to acclimate to ambient conditions. The water used in the petri dish was conditioned well water, kept at room temperature. Twenty minutes prior to testing, the larvae were fed *ad libitum* with brine shrimp. One performance recording was conducted for three larval minnows in each treatment. The test started after the fish acclimated to the petri dish and swam into the center portion of the grid, marked with a square, and paused. Once inside the center portion, the electronic chip was activated by pressing a trigger to elicit the predator avoidance response. The high-speed film recordings were saved as .AVI files and examined with the Image J program for Windows 7. The anterior tip of the head and posterior tip of the tail for each larva were marked, along with two dots on the one millimeter grid. The data was transferred to Microsoft Excel and used to calculate time of initiation (latency period), escape velocity (body lengths/ms during the first forty milliseconds after initiation), the total escape response (body lengths/(latency period in ms + 40 ms)), and the angle of predator avoidance escape (angle of bend after initiation of predator avoidance response) (Figure 1). The velocity was converted to body lengths per

millisecond to remove the effect of body size on predator avoidance performance. In order for the videos to be considered for analysis, each recording needed (1) to have a latency response of more than five milliseconds, (2) have their first response after predator stimulus be an escape turn (Liu & Fetcho, 1999), and (3) stay in the field of view for the length of the recording. The exclusion of some recordings that did not meet these criteria resulted in some differences among sample sizes between treatments.

Competitive Feeding Performance Assay

In this experiment, exposed minnows were tested against control minnows in a competitive feeding performance assay. This was a novel assay specifically developed for the current study with exposed fathead minnows competing directly with control minnows for a limited quantity of newly hatched brine shrimp. For this assay, the larvae were moved to the testing area at least twelve hours prior to testing and placed, in various combinations, into individual wells on a six well plate. Individual wells were 35 mm in diameter and contained 10 mL of conditioned well water. The 12 hr starvation period provided ample time of deprivation to empty the stomachs of the larvae without compromising their health. The combinations of treatments that were established included: two control larvae; control larva and 125 ng L^{-1} E1 low treatment larva; 625 ng L^{-1} E1 high treatment larva and control larva. One minnow in each test (assigned randomly) was stained with a calcein dye, which was identified using the SE-MARK detector by Western Chemical. The larvae were filmed using a Canon NTSC ZR100 camcorder during the assay. Before each test, the larvae were given 1 min to acclimate, since moving the wells into the camera's field of view could cause stress to the larvae. After 25-35

brine shrimp were added to a well, the larvae were given 1 min to consume as many brine shrimp as possible. After 1 min, the larvae were euthanized using a sodium bicarbonate MS-222 solution (230 g MS222, 115 g sodium bicarbonate, 1 L of well water). The remaining brine shrimp were then counted to determine how many brine shrimp the larvae in each well ate.

Statistical Analyses

All analysis of the data was performed in Prism version 4.01 (GraphPad Software Inc.). First the data for the three trials in the predator avoidance performance assay and the competitive feeding performance assay were averaged so that n=1 for each replicate. Next, the escape angle and percentage of brine shrimp eaten were arcsine transformed. Then data were assessed using a repeated measures one way ANOVA with a Geisser-Greenhouse's Epsilon treatment to adjust degrees of freedom for the F-distribution. This was followed by Tukey's multiple comparisons test. A p-value of less than 0.05 was considered significant.

Results

Larval Hatch and Survival

Looking at the hatching success, the percentage of larvae that hatch from fertilized embryos, the mean hatch success was 89.75% for control larvae, 95% for 125 ng L^{-1} E1 exposed larvae, and 93% for 625 ng L^{-1} E1 exposed larvae. From hatching to testing the mean survival percentage was 89.877 % for control larvae, 93.213% for 125 ng L^{-1} E1 exposed larvae, and 88.991% for 625 ng L^{-1} E1 exposed larvae.

Laval Predator Avoidance Performance

When tested twenty-one days post embryonic eyespot development, only the larvae exposed to 125 ng L^{-1} E1 had longer predator avoidance latency compared to the controls ($p=0.0095$) (Figure 9B). The latency period for the 125 ng L^{-1} E1 exposed larvae was twice that of the control larvae. The increased C-start latency period for the 125 ng L^{-1} E1 exposed larvae did not carry over into either a slower escape velocity ($p=0.8244$) or slower total escape response ($p=0.2257$). Exposure to E1 had no significant effects on body length, escape angle, escape velocity or total escape response (Figure 9).

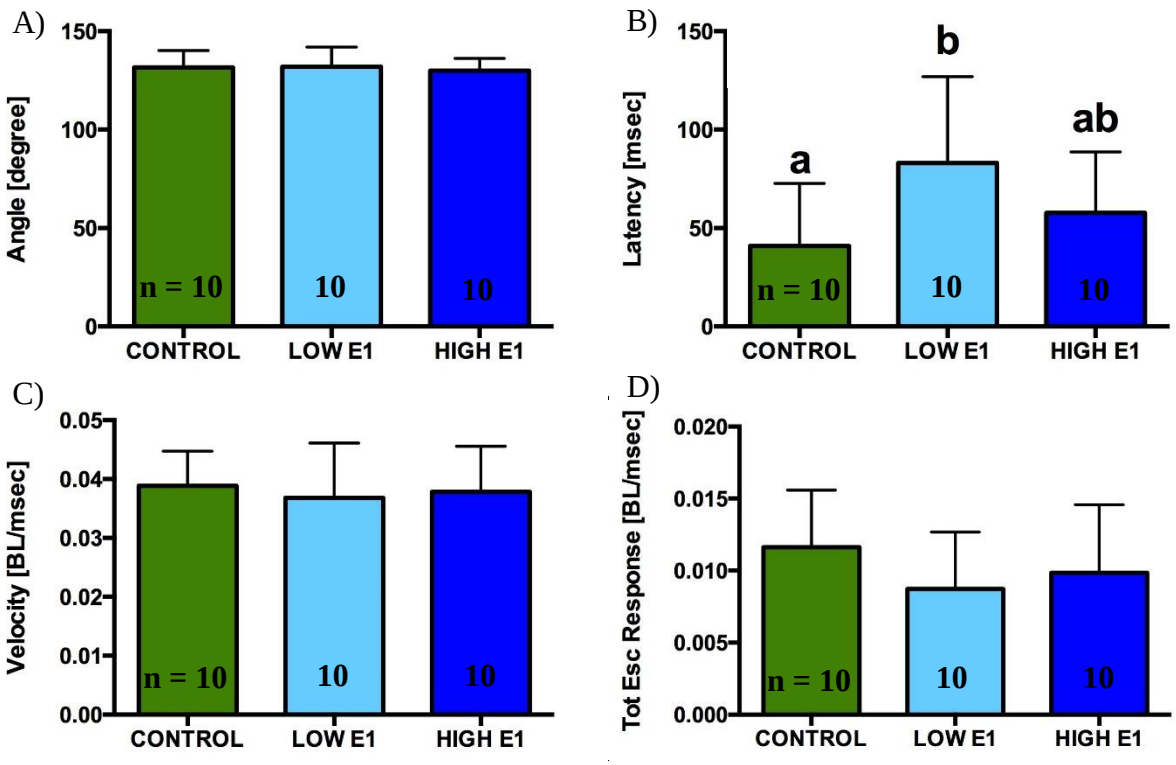

Figure 9: Effects of 21-day exposure to E1 on predator avoidance performance in larval fathead minnows. (A) mean escape angle, 180° minus the angle measured for farthest extent of bend; (B) mean of latency period, time in msec from initiation of stimulus, indicated by light turning on, to first head movement of predator avoidance performance; (C) mean escape velocity for 40 ms measured in body length per ms; (D) mean total escape response from stimulus until 40 ms post first head movement. These values combine latency period with escape velocity of predator avoidance response. Statistical significance was assigned when $p<0.05$ and indicated by lower cap letters. For treatments, ethanol solvent control (CONTROL), 125 ng L^{-1} E1 (LOW E1), and 625 ng L^{-1} E1 (HIGH E1).

Larval Feeding Performance

When tested twenty-one days post embryonic eyespot visualization, ethanol control vs. 125 ng L^{-1} E1 larvae ate significantly less brine shrimp compared to the paired ethanol control larvae (P=0.0175) (Figure 10). The wells with one ethanol control and one 625 ng L^{-1} E1 larvae did not differ in brine shrimp consumption from the paired ethanol control larvae (P=0.777).

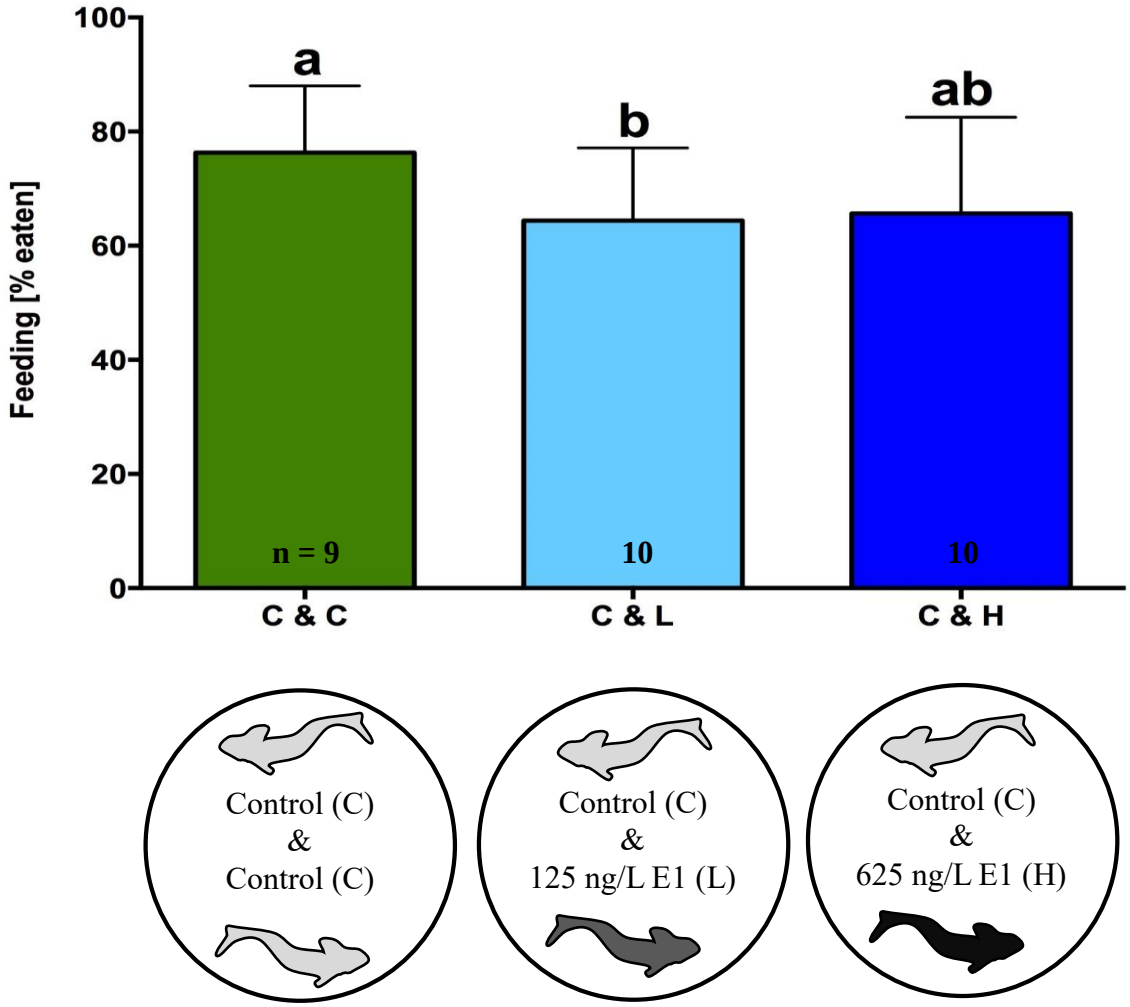

Figure 10: Effects of 21-day exposure to E1 on feeding performance in larval fathead minnows. Feed rate was determined by percentage of brine shrimp consumed in 1 min. Statistical significance was assigned when $p<0.05$ and indicated by lower cap letters. For treatments, ethanol solvent control (C), 125 ng L^{-1} E1 (L), and 625 ng L^{-1} E1 (H).

Chapter IV

DISCUSSION AND CONCLUSION

The central objectives of the two studies were to determine whether exposure of larval fathead minnows to environmental contaminants, such as pharmaceuticals or E1, diminish predator avoidance performance or feeding performance. In order for a larva to develop into adulthood and become sexually mature, the larva must succeed in avoiding predators and must consume adequate nutrients. Upon sensing a predation stimulus, the prey must respond rapidly, converting the stimulus to contraction of the lateral musculature, swimming quickly away; a delayed response or slowed reaction may lead to mortality. When a larva lacks feeding efficiency, it must devote significantly more time and energy to foraging. This depletes energy available for other biological conditions such as growth and predator avoidance. This may also lead to a larva spending substantially more time in open water, which would increase predator encounters, reducing their chances of survival to maturity.

In the investigation of larval exposure to pharmaceuticals or E1, larvae exhibited prolonged latency periods when exposed to diclofenac, rosuvastatin, or 125 ng L^{-1} E1 compared to a solvent control. However, the prolonged latency period was not accompanied by significant alterations in escape angle, escape velocity or total escape response. Interestingly, larvae exposed to all other pharmaceuticals and the 625 ng L^{-1} E1 treatment were not significantly different from control in any predator avoidance performance measure.

The assessment of predator avoidance performance can be separated into two components: reaction time (latency) and escape swimming velocity (escape performance). The results indicated that latency period was significantly increased for rosuvastatin, diclofenac, and 125 ng L^{-1} E1, while the escape performance did not differ significantly when compared to controls. These results are similar to those by McGee et al. (2009) and Painter et al. (2009), except that exposure to 50 ng L^{-1} E1 or 500 ng L^{-1} venlafaxine in their experiments resulted in a prolonged latency period and increased total escape response.

A delayed latency period may be the more crucial component of the total escape response when compared to the escape performance. With the size of predators being orders of magnitude larger than the prey, the escape performance may be negligible, suggesting that the latency period is the primary factor to avoid being captured by a predator during the larval stage (Rearick, et al., 2014). In other words, the larva's swimming speed is insufficient to outrun the predator, rendering its escape performance inconsequential and individual survival dependent on a short latency period. To illustrate, larvae that take little time distinguishing a predator stimulus and eliciting an escape response quickly may suffice in allowing the completion of the C-start and avoiding the predator. On a molecular level, the speed of events along the Mauthner signaling pathway determine the length of the latency period. The pollutants in our exposure may have delayed the predator stimulus from being perceived or delayed the signaling between the Mauthner cells firing to the motoneurons along the lateral musculature. A delay in the predator stimulus being perceived, may be a result of an altered threshold for excitation of the Mauthner cell. This would suggest that an increased stimulus, such as the predator physically being closer, may be needed to reach threshold and initiate response. Even with Mauthner cells functioning

normally, the signal from the Mauthner cells may be delayed to the motorneurons along lateral musculature.

The investigation of feeding performance of larval fathead minnows following exposure to pharmaceuticals suggests that larval foraging behavior is not affected by any of the pharmaceutical exposures. In contrast, European perch, *Perca Fluvitilis*, exposed to low (1.8 µg L^{-1}) and high (910 µg L^{-1}) treatments of oxazepam exhibited an increased feeding rate (Brodin, Fick, Josson, & Klaminder, 2013). This suggests that some pharmaceuticals are capable of affecting feeding efficiency. Even though our results did not produce an altered foraging efficiency, this does not necessarily imply that the pharmaceuticals tested will not alter foraging ability at concentrations other than the one tested in the present study. For example, when Japanese medaka fish, *Oryzias latipes,* were exposed to 1.0 mg L^{-1} diclofenac a diminished foraging efficiency was observed (Nassef, et al., 2010).

Larvae tested after exposure to 125 ng L^{-1} E1 consumed a smaller percentage of brine shrimp available compared to paired control larvae. Using the assumption that control larvae will have the same foraging efficiency whether competing with an exposed larva or another control larva, it appears that larvae exposed to 125 ng L^{-1} E1 exhibited a diminished foraging efficiency. Interestingly, a diminished feeding performance, similar to our 125 ng L^{-1} E1 results, has been observed when mummichog larvae, *Fundulus heteroclitus*, were exposed to 10 µg L^{-1} methylmercury (Zhou, Scali, Weis, & Weis, 1996); when grayling, *Thymallus thymallus,* were exposed to methylmercury between 0.27 mg L^{-1} and 3.8 mg L^{-1} (Fjeld, Haugen, & Vollestad, 1998), when Japanese medaka, *Oryzias latipes,* were exposed to either 6.15 mg L^{-1}

carbamezapine or 1.0 mg L^{-1} diclofenac (Nassef, et al., 2010), and when crucian carp, *Carassius auratus*, were exposed to sertraline between 4.36 µg L^{-1} and 116 µg L^{-1} (Xie, et al., 2015). This suggests that the larval stage is highly susceptible to contaminants, since various contaminants, each with a distinct mode-of-action, are able to alter the foraging efficiency of multiple species.

The ability of fish to forage efficiently affects growth and survival to sexual maturity. Therefore, feeding performance is ecologically relevant as it correlates to the overall fitness of an organism. Any impairment will increase the energy expenditure required to obtain adequate nutrients, while only sustaining minimal energy intake. As a result, overall fitness is decreased. More time spent foraging may also force the fish to spend more time in open water in order to capture the same amount of prey. This extra time in open water may increase predator encounters and probability of mortality.

One underlying mechanism that may explain the diminished feeding performance is the neurotransmitter serotonin (5-HT). Serotonin regulates many complex behaviors including feeding (Alanärä, et al., 1998). Future research should examine the relationship between pharmaceuticals, estrogens, serotonin, and feeding performance. A better understanding of the underlying mechanism of contaminant modes-of-action will allow us to establish safe limits of environmental contaminants.

The defined 21 day length of exposures in the current study may limit our ability to interpret the results. Examining both studies, the exposure duration does not take into account longer-term or life-cycle exposures. Future research should observe the long term effects of larval exposures to pharmaceuticals or E1. The long term effects could be additive or synergistic

with an increase of exposure length, causing a bioaccumulation of contaminants. It is known that bioaccumulation occurs over time (Zenker, Cicero, Prestinaci, Bottoni, & Carere, 2014). An example found in the aquatic toxicology literature quantified the bioaccumulation of oxazepam in muscle tissue of wild European perch, *Perca fluviatilis*, which was six times higher than the concentration of oxazepam present in the water (Brodin, Fick, Josson, & Klaminder, 2013). Not only is bioaccumulation a possible outcome of constant low levels of exposure, but biomagnification can occur as compounds are moved through the food chain. The increased concentrations in non-target organisms could become a human health concern if humans consume pharmaceutical-exposed fishes and potentially ingest therapeutic doses of various compounds. This may be of particular concern in populations that depend on fish as an important food source.

Another concern with pharmaceutical pollution relates to antibiotic resistance, an example being the compound sulfamethoxazole in the present study. Antibiotics are widely used in humans and veterinary medicine. These pharmaceuticals remain biologically active after consumption by either humans or livestock, entering the aquatic ecosystems either following transport through waste treatment facilities or as a result of runoff from farms. The low doses of antibiotics still have the capability to prevent some bacteria from growing, but the low doses may not persist long enough to cause mortality to the bacteria. This may lead to the bacteria mutating and becoming resistant to the antibiotic. Bacterial isolates examined from various organs of the catfish, *Clarias batrahus*, were observed to contain resistance to seven different antibiotics (Pathak & Gopal, 2005). Antibiotic resistance can be life-threatening to susceptible individuals especially the elderly and patients whose bodies have become immunocompromised, as they

must rely on antibiotics to overcome and eliminate bacterial infections since their own immune systems are inadequate.

Pharmaceuticals and estrogenic compounds are widely used and discharged into aquatic ecosystems (Koplin, et al., 2002; Richardson, 2012; Schultz, et al., 2011). With the increasing human population, increasing life expectancy, and an aging baby boomer population, the amount of pharmaceuticals used and discharged to the aquatic ecosystems will likely increase in the coming years. This increase of contaminants into the aquatic ecosystems could result in populations of non-target organisms to deplete and possibly face extinction. A similar effect has been observed in a whole lake study where exposure to ethynlestradiol resulted in a fathead minnow population decline (Kidd, et al., 2007; Palace, et al., 2009).

The effects of the pharmaceuticals and estrogens are not limited to non-target organisms in the aquatic ecosystems, but they can also affect terrestrial mammals, including humans. Discharged pharmaceuticals have been found in surface water, ground water and even drinking water (Schaider, Rudel, Ackerman, Dunagan, & Brody, 2014; Koplin, et al., 2002; Richardson, 2012). Although the concentrations are currently low and may have no effect on humans, increased concentrations with increased use of pharmaceuticals in the future could cause potential harm. Even with a daily consistent low dose exposure to these compounds, there is an increased chance of drug resistance. As a result, older individuals may need to take higher doses of pharmaceuticals to reach the same effect. This could be potentially dangerous since higher doses will increase the chance of toxic effects.

To conclude, we demonstrated that exposure to pharmaceuticals and E1 modifies both predator avoidance performance and feeding behaviors. Particularly, exposure to rosuvastatin (3,200 ng L^{-1}), diclofenac (1,600 ng L^{-1}), and 125 ng L^{-1} E1 prolonged the latency period during the predator avoidance performance. In addition, the exposure to 125 ng L^{-1} E1 diminished feeding performance. Future research should examine what concentrations of these pharmaceuticals and E1 instigate predator avoidance and feeding alterations. Pharmaceuticals and E1 have the potential to cause ecological consequences to both target and non-target organisms.

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