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Biological Consequences of Surface Water Pharmaceutical Contamination in the Fathead Minnow, Pimephales Promelas, and Bluegill Sunfish, Lepomis Macrochirus

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BIOLOGICAL CONSEQUENCES OF SURFACE WATER PHARMACEUTICAL CONTAMINATION IN THE FATHEAD MINNOW, PIMEPHALES PROMELAS, AND BLUEGILL SUNFISH, LEPOMIS MACROCHIRUS

by

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ABSTRACT

The field of Aquatic Toxicology has produced diverse studies on contaminants of emerging concern (CECs). Investigations have brought to light a plethora of knowledge on the subject; of particular interest are those involving pathways by which CECs arrive in waterways (source), the concentrations which they exist therein (fate), and to what extent they affect aquatic animals (effect). Particularly concerning CECs are pharmaceuticals that find their ways into aquatic environments, as these compounds are inherently biologically active and often stable in aqueous solution. Interest in potential adverse effects on exposed animals has begun to yield compelling evidence supporting behavioral and developmental disturbances in addition to physiological and anatomical pathologies. In an attempt to identify anomalies as a consequence of pharmaceutical exposure larval fathead minnows were examined in conjunction with the common panfish, *Lepomis macrochirus* (colloquially the bluegill sunfish), as models to build on prior evidence. The pharmaceuticals intended for investigation in my experiment are those representing multiple modes-of-action and were administered at concentrations found downstream of wastewater treatment plant (WWTP) effluent discharges. Fathead minnow exposed to 2000 ng/L diclofenac in a 21-day static exchange experiment were found to have inhibited foraging efficiency in a 60 second timed trial. Hepatocyte vacuole proliferation was found in bluegills exposed to methocarbamol (4000 ng/L) or sulfamethoxazole (2000 ng/L), while those exposed to a mixture of all pharmaceuticals or temazepam (2000 ng/L) experienced subdued plasma cortisol and hematocrit (temazepam only) concentrations.

ACKNOWLEDGEMENTS

For my parents, without whom this work would surely have been impossible.

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Chapter I: LITERATURE REVIEW

Contaminants of Emerging Concern

"Contaminants of Emerging Concern" can be broadly defined as any synthetic or naturally occurring chemical or microorganism that is not regularly monitored but has the potential to enter the environment and cause known or suspected adverse ecological and(or) human health effects. In practical terms, these chemicals are those which have been detected at potentially significant concentrations in various ecosystems and have limited previous research accomplished on their capacity to influence biological aspects of ecological environments. Examples which have been receiving increased attention in toxicology studies are endocrine-disrupting compounds (EDCs), polychlorinated biphenyls (PCBs), and pharmaceuticals (Flippin, Hugget & Floran, 2007, US Environmental Protection Agency, 2016, Wu et al, 2008).

These contaminants comprise a wide range of intended uses and arrive in aquatic environments via sources such as household waste, agricultural practices, and manufacturing processes. A cumulative list of contaminants identified to date would include hundreds of entries ranging from prescription and over-the-counter pharmaceuticals, to plasticizers and pesticides (Glassmeyer et al., 2005, Kolpin et al., 2002, Radjenovic, Petrovic & Barceló, 2007, Richardson, 2012). These chemicals are found in locations throughout the world at concentrations known to be capable of inducing adverse consequences in exposed wildlife species (Ai et al., 2010, Cha et al., 2005, Flippin, Huggett & Foran, 2006, Murata et al., 2011). Although the full spectrum of contaminants continues to be revealed, efforts to evaluate the impact of their presence

in surface water through laboratory and field studies are ongoing and continue to establish a knowledge base of detrimental effects in a variety of organisms (Wilkinson et al, 2016).

Pharmaceuticals as Contaminants of Emerging Concern

Placing responsibility for surface water pollution is a complex issue despite the obvious influence of large operations such as chemical manufacturing facilities or agricultural processes. While such sources have contributed to ecological and environmental damage, the deposition of many contaminants such as drugs and EDCs in surface waters is facilitated in part by increasing consumption by the general public and a historic proclivity for poor disposal habits. Indeed, the past several decades have yielded great progress in the field of pharmaceutical development with current estimates that up to 70% of the American population are using at least one prescription pharmaceutical (Mayo, 2015). Furthermore, prescription and usage figures in the United States are likely to rise in tandem with the age of the baby-boomer generation as medicinal treatment of age-related conditions precludes an increasing dependence on pharmaceuticals. While the presence of these pharmaceutical contaminants in surface water has been suspected for some time, identification and quantification efforts have historically been limited by a lack of analytical techniques capable of detecting the infinitesimal concentrations at which they exist in aquatic ecosystems. However, the advent of new technologies capable of bridging this gap has facilitated research aimed at establishing the true scope of the issue and necessity for concern (Wang et al, 2011, Wilkenson et al, 2016).

In the past few decades several research teams have canvassed surface water sites throughout the world and published their findings of detected contaminants. Often, the areas tested were those with increased susceptibility, such as those downstream from WWTP effluent (Kolpin et al., 2002, Radjenovic, Petrovic & Barceló, 2007, Richardson, 2012, Schoenfuss et al., 2015). These foundational studies revealed that pharmaceuticals are consistently found in the nanogram to microgram per liter range, although some figures are significantly higher (Phillips et al., 2010). In terms of human exposure, the potential for adverse health issues in those who come in contact with contaminated sources is minimal, as the consumption of water necessary for a single therapeutic dose would be impossible. However, the unique anatomical and physiological characteristics of aquatic species are such that even low concentrations of contamination results in constant exposure of certain tissues such as gills. Efforts to quantify pharmaceuticals in tissues of fish species after chronic exposure to environmentally relevant concentrations have elucidated uptake and depuration kinetics in tissues and support the need for further investigations into physiological and behavioral consequences (Schultz et al, 2011).

Exacerbated pharmaceutical usage on the global scale has yielded severe consequences which stretch beyond adverse biological implications for an exposed organism to include ecological and economic impacts. In fish species alone, laboratory exposure to environmentally relevant pharmaceutical concentrations has been linked to behavioral modifications (Brodin et al., 2013, Painter et al., 2009), disruption of reproductive systems (Cha et al., 2005, Flippin, Huggett & Foran, 2006), and pathologies in multiple tissues including the liver (Hoeger et al, 2005), kidney (Mehinto Hill, & Tyler, 2010), and gill (Schwaiger et al, 2004). Additionally, the potential for extensive

ecological effects is shown by the results of a whole-lake study of the estrogenic compound found in human birth control pills. In this study, researchers observed a total collapse of resident fathead minnow populations (Kidd et al, 2007). While these investigations are reflective of controlled settings, scenarios such as the near extinction of *Gyps* vultures in the Indian subcontinent are a testament to the scope of potential problems following unanticipated environmental exposure outside of a research project. Between 1990 and 2006 at least 95% of three species of *Gyps* vultures died of kidney failure (Meteyer et al, 2005) following ingestion of diclofenac-contaminated tissue of carcasses from livestock which had been treated with the drug (Green et al, 2004, Prakash et al, 2003). The die-off is estimated to have cost the Indian government $25 billion USD in medical costs as a result of increases in human contact with pest species (Vulture Conservation Foundation, 2016). In addition, a resurgence of previously suppressed animal-borne illness and disease were observed in response to booming populations of species with uncontested access to carrion previously consumed by the vultures (Markandya et al, 2008).

Pharmaceuticals of Interest

Due to the inherent size and complexity of the issue of surface water contamination the call for continued toxicology testing of both individual and mixtures of pharmaceuticals is one that must, and indeed continues to be, answered by a multitude of organizations throughout the world. By selecting a limited number of variables with relevance to their respective areas of expertise and local environments, researchers work to answer broad questions in small, targeted steps. This premise is the driving force

behind the following work which began by narrowing the list of possible pharmaceuticals to a manageable group reflecting prior laboratory experience, prominence in the environment, and representation of broad mechanistic classes. In utilizing techniques and species familiar to the field of aquatic toxicology, this research aims to investigate the biological significance of surface water contamination by diclofenac, methocarbamol, sulfamethoxazole, and temazepam, which are described in detail below.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and diclofenac are cyclooxygenase (COX) inhibitors commonly utilized for their mild analgesic capabilities in addition to inflammatory response moderation. In humans, inhibition of COX-1 has been shown to have a negative effect on cytoprotection, a result of which is an increase in potential for gastrointestinal (GI) ulcers due to inhibition of prostaglandin synthase activity and ultimately a reduction in mucosal defense (Wallace, 2008). Although less common, the incidence of drug-induced hepatic toxicity in human cases has also raised concerns (Boelsterli, 2003). Additionally, exposing cultures of murine central nervous tissue to diclofenac negatively impacts differentiation of neural stem cells into neurons and can cause apoptosis of these cells (Kudo et al., 2003). The toxicity of diclofenac is apparent in fish as well, with studies linking chronic exposure at low concentrations with altered developmental and reproductive cycles (Cha et al., 2005, Flippin, Huggett & Foran, 2006) and severe pathologies of gill and kidney tissues (Schwaiger et al., 2004, Triebskorn et al., 2004). Considering the conserved nature of COX enzymes across taxa (Chandrasekharan, 2004), it is imperative that further support and analysis of the mechanisms of NSAID toxicity are pursued in multiple model species.

Methocarbamol is a prescription-grade skeletal muscle relaxant prescribed for use in both human and veterinary practices, primarily for the treatment of muscle spasms (Mayo Clinic, 2015). Although the specific mechanism of action is not understood at this time, methocarbamol's primary relaxative and generally mild side effects such as drowsiness are thought to be the result of a central nervous system interaction with carbonic anhydrase (Parr & Khalifa, 1992). In addition, very little animal-based toxicological research has been accomplished on the drug aside from comparative efficacy analysis (Chou, Peterson, & Helfand, 2004). The combination of methocarbamol's behavior-influencing side effects, sparse toxicological history, and known concentrations in contaminated surface waters underline the necessity of its inclusion in additional research goals.

Temazepam is a benzodiazepine pharmaceutical closely related to the well-known behavior-modifying drugs oxazepam and diazepam. In humans, temazepam is prescribed primarily as a sleep aide although it has been shown to have additional uses such as treatment of anxiety and muscle spasms (Licata et al., 2011). Side effects common to benzodiazepine use are most commonly associated with lethargy, dizziness, and impairment of fine motor skills (Mayo Clinic, 2015). The mechanism of action of benzodiazepines in general is relatively well understood and involves depression of the central nervous system via manipulation of gamma-aminobutyric acid (GABA) receptor affinity (Roth & Roehrs, 1991). While little toxicological research has been published on temazepam itself, studies have shown that benzodiazepines are capable of influencing feeding and boldness behaviors in fish species (Brodin et al., 2009). Due to the highly conserved nature of GABA receptors in vertebrates and the lack of published work on

temazepam, continued investigation of its potential to induce adverse effects in organisms native to susceptible aquatic habitats may expand our understanding of the issue in unique ways.

Lastly, this study includes a sulfonamide antibiotic. Sulfamethoxazole is an analog of para-aminobenzoic acid (PABA) that is antibacterial in nature via its ability to prevent the synthesis of the B vitamin folic acid, a compound necessary for bacterial growth (The Merck Veterinary Manual, 2015). Due to the exogenous nature of folic acid in humans and animals alike, use of the antibiotic in both populations became widespread after its introduction in 1961 (Sittig, 2007) with prescriptions commonly given for a variety of bacterial diseases including MRSA and strep-throat. Commonly reported side effects are generally mild in nature and often relating to GI tract distress (The Mayo Clinic, 2015). Considering the potential for disruption of intestinal bacteria populations (Mayo Clinic, 2015), it is conceivable that abnormalities within the GI tract as a result of chronic exposure may be detrimental to the overall health of exposed organisms. Studies in multiple countries have identified antibiotic CECs ubiquitously (Kolpin et al., 2002, Murata et al., 2011, Ai et al., 2010, Richardson, 2012). In Japan, median concentrations were found as high as 7.3 ng/L, with sulfonamides as the dominant pharmaceuticals in surface waters near large agricultural operations, although higher concentrations were noted in urban areas despite lower usage (Murata et al., 2011). Interestingly, Ai et al. (2010) found Chinese mariculture operations contributed to contamination concentrations even more than urban and agricultural sites, and of river and marine samples examined, sulfamethoxazole had the highest concentration of all antibiotics with values as high as 66.6 ng/L and 25.2 ng/L respectively. While the widespread use and subsequent detection

of sulfamethoxazole in water bodies has prompted preliminary investigations of potential toxicological effects (Johansson, Janmar & Backhaus, 2014, Kim et al., 2007), additional work is necessary to further elucidate possible adverse consequences in exposed species.

In addition to the assessment of each of these pharmaceuticals in a standalone fashion, it is important to factor in the potential for each to influence the others in simultaneous exposure situations such as those occurring in the field. Considering the range of pharmaceuticals consumed by the human population, the innumerable biological mechanisms by which they function, and the understanding that usage contraindications exist, the potential for mixtures of contaminants to induce results which are dissimilar to those from individual compounds is of concern. Recent side-by-side investigation of the effects on fathead minnows of complex mixtures in a field site as compared to those of in a laboratory setting have yielded disparities in results (Schoenfuss et al, 2015). Such findings lend credence to the necessity of addressing the potential interaction of variables in contaminant toxicology testing as an addition to single-compound methodology.

The Utility of a Multi-Faceted Approach to CEC Exposure Investigation

An aspect of toxicological research that is vital to the goal of developing a well-rounded understanding of their environmental impact is the need for studies which incorporate effects at multiple life stages of exposed organisms. While there is no question as to the value in examining consequences of single life-stage exposure, the utility of more expansive experiments is in the inherent ability to better reflect the reality of contaminated habitats as they exist in the wild. In this regard an attempt can be made to identify and relate deleterious effects such as those of physiological developmental

stages to reproductive abnormalities or population dynamics. Research projects designed to simultaneously investigate multiple effects benefit from a reduction in variables that may prove unavoidable in separate examinations. With this in mind, the following study was designed to incorporate the support of both larval and adult stage analysis. Ultimately, the goal of this work is to address the "effect" step of an investigation in a two-fold manner: by assessing the potential for select pharmaceuticals of interest to adversely impact subject behavior as a result of early life exposure in larval fathead minnows, and by identifying anatomical and physiological anomalies in bluegill sunfish as a result of mid-life-stage exposure.

Chapter II: LARVAL FATHEAD MINNOW EXPOSURE

THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*

The fathead minnow, *Pimephales promelas* are freshwater teleosts known as both a bait fish and a model species in aquatic toxicity testing (Ankley and Villeneuve, 2000, USGS, 2016). As minnows they are characteristically small with adults typically ranging from 20 to 50 mm in length (Danylchuck, Tonn, & Paszkowski, 2011) and generally exhibit a coloration gradient progressing from white or silver ventrally to a gray or slightly green dorsally. However, sexual maturity and social hierarchy are known to influence variations in size and color (Ankley et al, 2001), and morphological variation has been noted between individuals from different geographic locations (Danylchuck, Tonn, & Paszkowski, 2011). Differentiation of the fathead from similar species such as creek chub or bluntnose minnow is thereby a difficult task involving comparisons of species-specific scale patterns, fin rays, and mouth geometry.

P. promelas are thought to be native to a geographic region spanning a portion of south eastern Canada and the eastern half of the United States (Danylchuck, Tonn, & Paszkowski, 2011, United States Geological Survey, 2016), although they are currently found throughout most of North America, parts of Europe, and Asia (USGS, 2016) as a result of both intentional and accidental introduction (Pflieger, 1975). The ability of populations to proliferate in such a broad range is due in part to the fathead minnow's tolerance to normally prohibitive water quality conditions such as low pH and oxygen (Ankley and Villeneuve, 2000, Danylchuck, Tonn, & Paszkowski, 2011). As a consequence, fathead minnows are common in various lentic and lotic water bodies

ranging from large lakes and rivers to small ponds, streams, and even drainage ditches (Duffy, 1998, Danylchuck, Tonn, & Paszkowski, 2011, Paulson & Hatch, n.d., Schultz et al, 2011). However, individuals are most likely to be found occupying areas with warm, shallow water and access to spawning sites (Danylchuck, Tonn, & Paszkowski, 2011).

In addition to their tolerance of harsh conditions, fathead minnows are omnivorous and capable of surviving in environments with limited foraging options (Danylchuck, Tonn, & Paszkowski, 2011), a feature which lends additional support to their ecological adaptability. In general, aquatic invertebrates, algae, plants, and detritus are the primary components of a fathead diet (Duffy, 1998). Fathead minnows are also important to aquatic ecology as a source of prey for piscivorous species such as pike and walleye (Duffy, 1998), and have been introduced in many areas as a source of food for game fish (Danylchuck, Tonn, & Paszkowski, 2011, Duffy, 1998).

The life of a fathead begins after an incubation period of approximately 4 to 5 days (Duffy, 1998). In the ensuing months larvae experience rapid growth as they develop through several defined stages characterized by the expression of morphological features specific to each phase (Danylchuck, Tonn, & Paszkowski, 2011). After five to six months individuals mature which is reflected in males by the exhibition of secondary sexual characteristics including a dorsal pad, dark vertical coloration banding, and the development of keratinous tubercles on the snout and lower jaw (Ankley & Villeneuve, 2006, Ankley et al, 2001, Wynn-Edwards, 1932). In females, sexual maturity is less obvious from a physical perspective although it is marked by the development of an ovipositor which is used to place clutches of eggs in nests (Ankley et al, 2001). Nest sites can be of varying sizes and material compositions yet typically have a semi-flat underside

surface (Wynn-Edwards, 1932). This willingness of fathead minnows to utilize artificially provided objects is of great value to researchers who use the species laboratory settings. A female fathead will spawn several times during the breeding seasonal when water temperatures are maintained at or above approximately 18° C (Duffy, 1998). This behavior is known as fractional spawning and allows females to place up to several dozen clutches of eggs in one season, each containing as many as several hundred eggs (Duffy, 1998, Thomsen & Hasler, 1944).

The utility of the fathead minnow as a model species in scientific research has developed over a century of laboratory use most commonly to investigate the toxicity of environmental contaminants (Ankley & Villeneuve, 2006). Continued use in this regard is due to the well documented natural history of the species including behavioral aspects such as nest defense and reproduction (Paulson & Hatch, n.d.), ability to reproduce in artificial settings (Ankley & Villeneuve, 2006), and physiological characteristics such as tolerance of varying water quality conditions (Denny, 1987, Duffy, 1998, Sommer, 2011). In addition, the establishment of extensive rearing and handling protocols, consistent availability from aquatic laboratory species suppliers, and relatively low cost are benefits which have lent themselves to the continued use of the fathead minnow in all sectors of aquatic research (Denny, 1987, Ankley & Villeneuve, 2006).

Predator Avoidance Behavior

In the wild, prey species such as the fathead are highly susceptible to predation by piscivorous fishes (Danylchuck, Tonn, & Paszkowski, 2011, Duffy, 1998). However, many species have developed physiological and behavioral predator avoidance abilities.

An example of such is the use of the chemical alarm signal known as Schreckstoff which is produced by a majority of freshwater fish species when they are attacked (Smith, 1992). Nearby fish are capable of detecting the chemical after its release from damaged skin tissue and have been shown to respond by expressing behaviors such as hiding and erratic movement (Mathis & Smith, 1993) to minimize risk of predation. Research aimed at understanding these predator avoidance behaviors in fish has also identified an avoidance technique characterized by the contraction of the subject's body into a C-shape after encountering a predatory stimulus and prior to rapidly propelling away from it (Eaton, Lee, & Foreman, 2001, Nissanov & Eaton, 1989). Known as a C-start, this behavior has since been found to rely on a complex series of defined physiological events (Eaton, Lee, & Foreman, 2001), and is vital to the ability of an individual to react to sensory stimuli perceived to be predatory in nature.

From a physiological perspective, C-start reactions in a fathead minnow begin with the detection of predatory stimuli and activation of specialized nerve cells which develop in the hindbrain during early larval stages (Palande, 2011, Sillar, 2009). The excitation of these Mauthner cells results in the unilateral contraction of dorsolateral musculature and characteristic bending of the body into a C-shape (Eaton, Lee, & Foreman, 2001, Nissanov & Eaton, 1989). The ensuing release of contracted muscle facilitates a rapid acceleration over a short distance. The execution of a C-start is a well-documented neurochemical response with an average start-to-finish timeframe of as little as 7 to 12 ms (Eaton, Lee, & Foreman, 2001). However, exposure to pharmaceutical contaminants has been shown to increase reaction times in previous studies (McGee et al., 2009, Painter et al., 2009). These methods may be used to further our understanding

of how pharmaceutical exposure influences the development and expression of innate behaviors in larval fishes (McGee et al., 2009, Painter et al., 2009). Ultimately, the implications of an impaired predator avoidance response may be far reaching, with potential to disrupt delicate habitats which balance on predator-prey relationships.

Feeding Behavior

Similar to behaviors involved with individual self-preservation, population health and mortality are inextricably linked to foraging ability of constituents. However, unlike the "preprogrammed" (innate) reaction of a C-start, foraging skills of the fathead minnow are influenced in part by learning from experience (Kieffer & Colgan, 1992). During feeding, individuals hone their abilities by progressing from accurate identification to active engagement of a food source, both of which are cognitive and implicitly reliant on CNS functions. Not surprisingly, aquatic contaminants such as pharmaceuticals, which are capable of bypassing the blood-brain barrier and acting on nervous tissues, have been shown induce quantifiable disturbances in feeding behavior (Brodin et al., 2009). In this regard feeding efficiency assays have been established as a unique way of evaluating the potential for pharmaceutical contaminants to influence the dynamics of aquatic ecosystems via behavioral modification of their habitants (Hedgespeth, Nilsson & Berglund, 2014).

METHODS AND MATERIALS

Experimental Design

1-day old fathead minnow larvae were shipped overnight to the Aquatic Toxicology Laboratory at St. Cloud State University (St. Cloud, MN) from a laboratory supplier (Environmental Consulting and Testing, Superior, WI). After a period of acclimation, random groups of 20 individuals were placed in one of 24 total glass mason jars with 4 jars designated and labeled for each of the 6 treatments: Diclofenac (2000 ng/L), Methocarbamol (4000 ng/L), Sulfamethoxazole (2000 ng/L), and Temazepam (2000 ng/L), Mix-All (all preceding pharmaceuticals at their respective concentrations), and Control (Ethanol, 2000 ng/L). Jars initially contained 500 mL ground water, with 250 mL being replaced with pre-mixed treatment water every day during the 21-day exposure. Due to the limited number of fish able to be analyzed on a single day, 12 randomly selected jars began receiving treatment water on experiment day 0 and the remaining jars on day 1. Consequently, in the first exposure the first 12 jars to receive treatment water had an experimental timeline of October 15 thru November 5th, 2015, with the subsequent jars from October 17th. thru November 7th. The duplicate exposure was conducted in a similar fashion with timelines of November 18th thru December 9th and November 19th thru December 10th, 2015, respectively.

Experimental Procedures

Jars were kept on a 16 h light/8 h dark cycle in an 0.5m x 1m laminar flow hood at room temperature. All treatment groups were fed a diet of laboratory-hatched brine shrimp (San Francisco Bay Brand, San Francisco, CA) *ad libitum* twice daily. Static

water exchanges were accomplished daily using syphons to drain half of the water in each jar before manually replacing with newly prepared treatment water.

Exposure Chemicals

Treatment water was pre-mixed using concentrated solutions of each pharmaceutical in 100% ethanol measured into 1.0 L plastic bottles containing ground water from a dedicated well at St. Cloud State University. Bottles were stored frozen at -40° C prior to the experiment and contents allowed to reach room temperature before use.

Water Quality Analysis

Water removed from the 4 jars in each treatment group during exchanges was combined and analyzed using a YSI model 556 MPS (YSI Co., Yellow Springs, OH) to record pH, salinity, conductivity, dissolved oxygen, and temperature. Chlorine, hardness, and alkalinity concentrations were periodically recorded using AquaChek 5-in-1 Water Quality Test Strips (Hach Company, Loveland, CO.).

Predator Avoidance Assay

In order to visualize C-start reactions, previously established protocols (McGee et al., 2009, Painter et al., 2009) were followed. A single FHM larvae was placed in a 5 cm diameter clear glass arena containing 10 mL ground water. The area was placed on a 1mm x 1mm paper grid and the test subject allowed to acclimate for several minutes. At a point when the larvae came to a stop in the center of the arena, an artificial predator stimulus was trigger-activated via a vibrating chip beneath the arena. The ensuing

avoidance behavior was recorded using a high-speed (Redlake M1 Digital High-speed) camera mounted 50 cm above the plate, capturing 1000 frames per second. Video recordings were captured and stored in VFI format. The procedure was repeated with three individuals from each jar. After each trial the subjects were transferred to a holding well and euthanized with a solution of MS222 in a sodium bicarbonate and groundwater solution. All carcasses were subsequently preserved in 1.0 mL centrifuge tubes containing and stored at -80°C for further analysis.

Predator Avoidance Assay Analysis

Analysis of the C-start video files was accomplished using ImageJ software (National Institute of Health, Bethesda, MD). Four endpoints were recorded from each video file including latency period, escape velocity, total escape response, and angle of escape. These can be defined as the time between activation of the stimulus and initial movement of the subject's head, the velocity of the larvae in the first 40 msec after the latency period ends, total velocity in the 40 msec after the stimulus begins, and the two-dimensional difference in angle between the orientation of the subject pre and post stimulus, respectively. In order to normalize data to account for varying sizes of the larvae, velocity and total velocity were measured in body lengths per msec.

Feeding Rate Assay

To examine variations in feeding behavior between treatment groups, three pairs of fathead minnow larvae from each treatment jar were randomly transferred to 12-well plates containing 10 mL ground water in each well. Fathead minnows in these wells were

subject to fasting and acclimating overnight prior to the assay. 25-35 brine shrimp were manually counted under a microscope before being placed in the wells with the larvae. Larvae were given 60 seconds to feed before being euthanized via the addition of 1 mL of MS222 solution to the well. All carcasses were preserved in 1.0 mL plastic vials and stored at -80°C.

Feeding Rate Assay Analysis

The number of brine shrimp remaining in each well after removal of the larvae was assessed and recorded using a Leica DMZ750 light microscope. Results were identified as percent consumption.

Statistical Analysis

Data was tested for normality using Dunnett's posttest. Unpaired T-test comparisons of C-start and feeding behavior endpoint data from each treatment group against the control group was accomplished in Microsoft Xcel 2013 (Microsoft Corporation, Redmond, WA). Additionally, angle of escape and percent consumption data were arcsine transformed prior to analysis (Sokal & Rolf, 1981).

RESULTS

Mortality and Water Chemistry

A statistically significant ($p < 0.05$) variance was found in survival between the diclofenac, methocarbamol, temazepam, and sulfamethoxazole treatment groups and the EtOH control group (Figure 2.1)

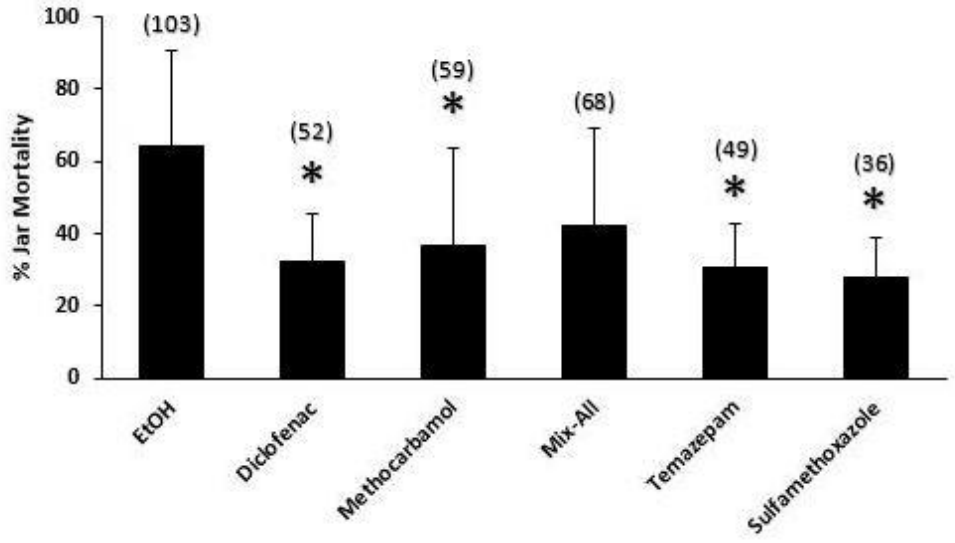

Figure 2.1. Average survival of *P. promelas* experienced after 21-day treatment exposure; significant difference ($p < 0.05$) between EtOH control group and diclofenac, methocarbamol, temazepam, and sulfamethoxazole groups; methocarbamol concentration of 4000 ng/L, all others at 2000 ng/L; error bars indicate one standard deviation; numbers in parentheses indicate *n* value; unpaired T-test.

Predator Avoidance Endpoints

No statistically significant ($p < 0.05$) differences were found in latency, initial velocity, total velocity, or angle of escape between treatment groups and the EtOH Control (Figure 2.2a, b, c, d).

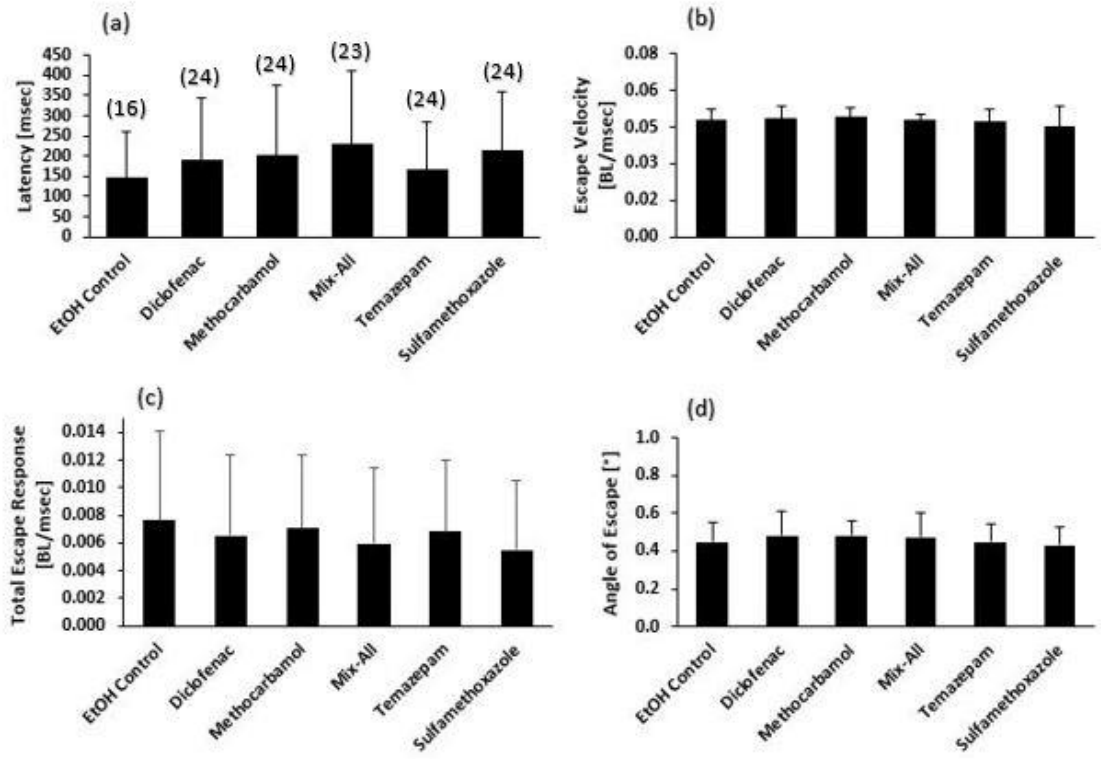

Figure 2.2. Analysis of *P. promelas* C-start response to artificial predatory stimulus in terms of averages in endpoints: latency (a), escape velocity (b), total escape response (c), and angle of escape (d) after 21-day exposure to pharmaceutical contaminants; no significant differences ($p < 0.05$) between treatment groups and the ethanol control group; error bars indicate one standard deviation; numbers in parentheses indicate *n* value of trials; unpaired T-test; angle of escape is arcsine transformed.

Feeding Behavior Results

The Diclofenac treatment group had a significantly lower ($p < 0.05$) average percent consumption of brine shrimp as compared to the EtOH control treatment (Figure 2.3). No other treatment groups varied significantly from the EtOH control.

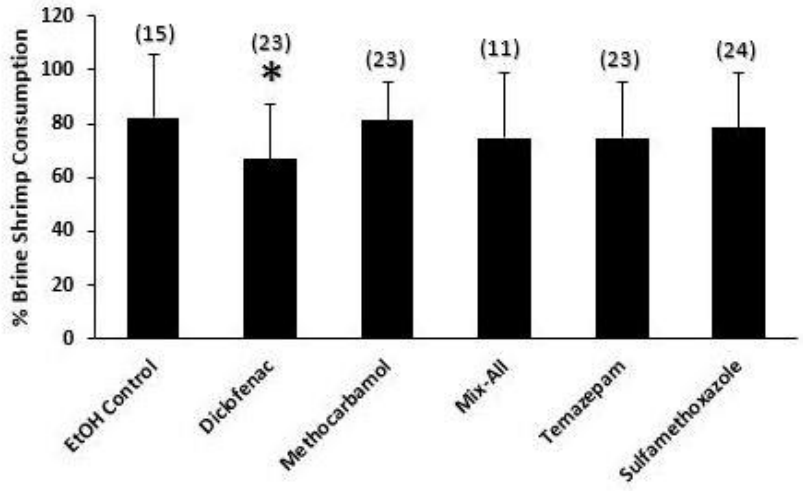

Figure 2.3. Feeding efficiency of *P. promelas* represented as averages of percentage of total brine shrimp consumed 60-second feeding trials; error bars indicate one standard deviation; numbers in parentheses indicate *n* value of trials; significant difference ($p < 0.05$) between treatment groups and the ethanol control group found only in diclofenac; unpaired T-test; all data is arcsine transformed.

DISCUSSION

The objective of this study was to evaluate the potential for pharmaceutical surface water contaminants to influence the expression of behaviors in fathead minnow larvae as a result of early life stage exposure to pharmaceuticals at environmentally relevant concentrations. We predicted that the biological consequences of such exposure would be manifested in a modulated response to predatory stimulation (C-start endpoints). We also predicted that fathead minnow larvae which experienced any of the same exposures would exhibit a significant deviation in feeding efficiency as compared

to control treatment peers. While the data acquired from these experiments did not support our predictions to their full extent, we did find evidence that such methodology may be useful in toxicological investigations of pharmaceuticals.

The most notable discrepancy between treatment groups and the control group is the survival of larvae over the 21-day exposure period. While the treatment jars experienced an average mortality below 45%, the average ethanol control jar mortality was 64% (Figure 2.1). This finding is particularly interesting due to its support of hypotheses which would implicate the treatment pharmaceuticals in either providing a therapeutic effect which in some way inhibited mortality rates, or in depressing fungal growth rates which also facilitates increased likelihood of survival in our experimental conditions. The lack of surviving control treatment subjects at the time of behavioral assay is a source of concern for the accurate comparison of endpoint data between treatment groups and necessitates modifications to methodology in future studies.

None of the pharmaceutical treatments had any significant effect on C-start performance. This is perhaps the most surprising of the results data, as previously published investigations of behavioral modification have found discrepancies between fish exposed to pharmaceuticals such as diclofenac and oxazepam, a benzodiazepine similar to temazepam, at similar concentrations (Brodin et al, 2013, Nassef et al, 2010). This could be explained by the discrepancy in mortality rates between treatment groups and the control group, as the few surviving control individuals may not accurately represent the average performance data of a larger population.

Despite the lack of statistically significant findings, the degree of variation in average latency and total escape response endpoints is of interest and supports the need

for continuation of behavioral modification testing. In this context it may be worthwhile to modify existing methodology to mitigate mortality rates by protecting subjects from fungal growth, as we believe this was the cause of nearly all deaths during the exposure timeline.

In the feeding efficiency assay, the only variation between the ethanol control larvae and the pharmaceutical treatment groups was a significant reduction in average percent consumption by those exposed to diclofenac at 2000 ng/L (Fig. 2.3). This finding is similar to that of other studies which have investigated the adverse effects of diclofenac on feeding behavior in several organisms (Nassef et al, 2010, Eades & Waring, 2010). The physiological mechanism by which an NSAID inhibits foraging efficiency is likely complex in nature and may be the manifestation of one or several underlying factors such as disruption of neurotransmitter pathways, motor function deficits, and anatomical pathologies Fent et al, 2006, Hoeger et al, 2005, Mehinto et al, 2010, Nassef et al, 2010, Eades & Waring, 2010, Schwaiger et al, 2004, Zeng et al, 2009). Foraging behavior in aquatic organisms has been studied extensively and is thought to be representative of an individual's swimming performance (De Lange et al, 2006, Zeng et al, 2009). Moreover, both foraging efficiency/behavior and swimming performance of an individual organism are integral to its relative survival chance (Taylor & McPhail, 1986). Such parallel and interrelated findings highlight the value of further investigation into the implications of aquatic contaminants on population health.

The lack of significant findings between the ethanol control larvae and the methocarbamol, mix-all, and temazepam treatment groups is also of interest. Despite methocarbamol and temazepam have different functions (muscle relaxant and sleep aide,

respectively), ingestion of either is frequently reported to be accompanied by side effects such as lethargy and drowsiness. Considering the level of physiological complexity and reliance on accurate motor function inherent to both predator avoidance and foraging behaviors, the lack of significantly subdued endpoint data for both pharmaceuticals is surprising. This is especially so in light of previous toxicology studies on these and other chemicals with similar mechanisms of action and side effects which describe effects of exposure in altered swim speed, foraging efficiency, and boldness behaviors (Brodin, Fick, Josson, & Klaminder, 2013, De Lange et al, 2006, Nassef et al, 2010). There are several possible explanations for the absence of significance in these treatments, including a more rapid development of resistance than with other chemicals, a modulating effect between chemicals in the same mixture, and/or abnormal variance in individual tolerance within the populations.

Chapter III: ADULT SUNFISH EXPOSURE

THE BLUEGILL SUNFISH, *LEPOMIS MACROCHIRUS*

Lepomis macrochirus (colloquially the bluegill) is a freshwater species native to a broad region of east central North America spanning south and east from the Great Lakes area (Nelson, 2006, Parr, 2013, USGS, 2013) and overlapping broadly with native fathead minnow populations. Similar to the fathead minnow, sport fishing has resulted in a greatly broadened distribution of the species, although in the case of the bluegill populations outside of the native range are generally a result of intentional stocking (Mecozzi, 2008, Nelson, 2006). Like many of the species prescribed the name "panfish" the bluegill has a semicircular, laterally compressed body with a narrow caudal peduncal, dark blue-green dorsal coloration, and brighter orange to yellow ventral hues (Mecozzi, 2008, Parr, 2013). However, the bluegill has a chromatically unique feature in its dark blue colored lower jaw and opercular flap (Mecozzi, 2008, Willis, 2005).

Wild bluegill are known for being nest builders with a penchant for warm, shallow waters in lakes and ponds or slow moving waterways (Mecozzi, 2008, Parr, 2013, Willis, 2005). Their diet is mainly composed of aquatic invertebrates such as worms, crayfish, and insects. Although adult bluegill are known to consume other fish species, their carnivorous tendencies are anatomically constrained, limiting prey to minnow species and very young fish (Mecozzi, 2008). Natural predators of the bluegill include large pisciverous species common to lakes and rivers of their natural habitat as well as birds of prey and carnivorous mammal species (Parr, 2013).

In the spring, populations tend to migrate towards shallow areas susceptible to increased temperatures such as inlets from nearby rivers and streams, and shaded or weedy locations near shorelines (Mecozzi, 2008). Such proclivity may be indicative of an ecological trap unique to species with similar migratory traits, as seasonal meltwaters are known to facilitate surges of increased contaminant concentrations (Loraine & Pettigrove, 2006). Prolonged occupation of these aquatic habitats with lower than normal water quality conditions may lead to the onset of adverse biological consequences more readily than species which primarily reside in less susceptible waters and thus experience a lesser degree of contaminant exposure. This ultimately makes the bluegill an indicator species, as fluctuations in population health reflect contaminant load in in-flowing waterways in proximity of seasonal breeding grounds.

During the late spring breeding window adult male sunfish build nests to attract females and are known for their tendency to aggressively defend them from competing males (Mecozzi, 2008, Parr, 2013, Willis, 2005). Eggs laid and fertilized in the midsummer spawning months typically hatch after 2 to 5 days of incubation and the resulting fry reach sexual maturity in anywhere from 2 to 7 years (Mecozzi, 2008, Parr, 2013). Although figures can vary depending on geographic location, in favorable environmental conditions fully grown *L. macrochirus* are known to reach an average mass of around 200 grams, with a total length limited to the 20 centimeter range (Mecozzi, 2008, Parr, 2013).

Similar to the fathead minnow, the bluegill has a history of use as a model species in the assessment of aquatic contaminants (Nelson, 2006). Their continued usage such is facilitated by a tolerance for laboratory settings including fluctuating water quality

conditions and willingness to reproduce in artificial habitats. Additionally, despite the prolific nature of the species and ubiquity of populations in freshwater habitats throughout much of North America, bluegill are thought to have limited home range (Mecozzi, 2008). This characteristic is thought to lend itself to their utility as indicators of localized surface water contamination, as accumulation of contaminants in bluegill tissues is often more readily quantifiable than from species which are more likely to avoid early exposure due to migratory habits.

METHODS AND MATERIALS

Experimental Design

Twelve 60 L aquaria were arranged in a side-by-side 3 x 2 design with two tanks per treatment. Adhesive paper was placed on external sides facing neighboring tanks to prevent social interaction of treatment groups, and holes drilled on lateral sides for drainage. Covers were fashioned from Plexiglas to prevent escape while also facilitating feeding, the use of air stones, and inflow from the water source. Source ground water from a dedicated well at St. Cloud State University was maintained at 21.5°C in a stainless steel head tank and drained to 6 stainless steel secondary mixing tanks located above the aquaria. A Masterflex 7523-40 peristaltic pump (Cole-Palmer, Vernon Hills, IL) was used to dispense diluted stock solutions of each treatment into the mixing tanks from glass containers. Individual aquaria were gravity-fed the contents of mixing tanks at a flow-thru rate of 200 mL/min. Each tank was stocked with approximately 48 randomly sorted juvenile hybrid bluegill sunfish from a laboratory supplier (10,000 Lakes Aquaculture) on August 4th, 2015. It is worth mentioning that during the initial sorting

process the methocarbamol treatment tank was accidentally given 50 fish and the mix-all tank was only given 47. The exposure ran for a total of 31 days between the dates of August 6th, and September 5th, 2015.

Experimental Procedures

Aquaria were kept on a 16 h light:8 h dark schedule. Fish were fed an *ad libitum* diet of frozen brine shrimp and blood worms (Brine Shrimp Direct, Ogden, UT) twice daily. Mixing tank inflow rates from the head tank were maintained at 400 mL/min via flow regulators and outflow to aquaria through 5 mm diameter tubing at 200 mL/min. Flow rates to and from the mixing tanks as well as from the peristaltic pump were monitored daily and adjustments made as needed to promote consistent flow rates throughout. Treatment solutions were prepared on a daily basis by mixing 1 mL of stock chemicals with 10 L ground water in glass containers to meet concentrations identical to those listed in the fathead minnow exposure. Black paint was applied to the exterior of the containers and tinfoil covers were used to limit light exposure. The time and remaining volume in each container was recorded during daily exchanges of treatment water.

Water Quality and Chemistry

Water quality measurements were recorded daily using a YSI model 556 MPS (YSI Co., Yellow Springs, OH) for temperature, pH, and dissolved oxygen (Table 1.1). Samples taken from each mixing tank on a recurring basis (approximately every 3 days) were stored in amber glass vials before being shipped frozen to the United States

Geological Survey (USGS) Water Chemistry Laboratory in Denver, Colorado for water chemistry analysis.

Table 3.1: Average and standard deviation of daily recorded temperature, dissolved oxygen, and pH readings from adult bluegill sunfish exposure tanks.

	Temp. (°C)	DO (mg/L)	pH
Avg.	21.05	7.49	7.66
St. D.	± 0.22	± 1.77	± 0.15

Data and Tissue Collection

Dissections occurred on days 16, 18, 30 and 31, with 8 from each tank in each event. Anesthesia was accomplished by transferring the fish to a vessel containing MS222 in a sodium bicarbonate buffered ground water solution. Prior to dissection, the mass, standard and total lengths of each individual were measured (0.01 g precision, Taylor Stainless Steel Food Scale, Taylor Precision Products, Oak Brook, IL). During dissections, gill, liver, kidney, intestine, and gonadal tissue were harvested, placed in plastic histological cassettes (Tissue-Loc Histoscreen Cassettes, Thermo Scientific, Kalamazoo, MI), and stored in a 10% buffered formalin solution prior to chemical dehydration. Total, liver, and gonad masses were recorded for analysis of morphometric and organosomatic indices (0.001 g precision, Mettler Toledo AG245, Columbus, OH). Brain tissue was harvested and placed in 1.0 mL centrifuge tubes containing RNA Later (Sigma Aldrich, St. Louis, MO) and stored at -80°C. Blood from the caudal vasculature was collected for evaluation of blood glucose concentrations (BGL), hematocrit, and plasma cortisol concentrations.

Morphometric and Organosomatic Indices

One morphometric and two organosomatic indices were calculated from specimen recordings. Body condition factor (BCF) is an evaluation of the organism's total mass in relation to its total length (total mass/total length^3) (Fulton, 1904). In a similar fashion, hepatosomatic (HSI) and gonadosomatic indices (GSI) are a reflection of liver and gonad health and maturity, respectively, and are calculated by evaluating recorded organ mass as a fraction of an organism's total mass (organ mass/total mass x 100).

Histopathology

Tissues harvested for histopathological analysis were dehydrated using established protocols (Carson, 1997) and embedded in paraffin. Liver, kidney, and gonad samples were sectioned at approximately 5 µm and gills at 7 µm (Olympus Cut 4055 Microtome, Olympus America Inc., Center Valley, PA). All slides were stained with a standard hematoxylin and eosin stain (Gabe, 1976, Carson, 1997) using a Leica ASP 300 Automated Tissue Processor (Leica Camera AG, Wetzlar, Germany).

Liver vacuolization was assessed on a scale of 1 to 4 where a 1 reflects a complete lack of cellular vacuolization in the visible cells, a 2 indicating vacuolization was present in less than 25%, a 3 indicating between 25% and 50% vacuolization, and a 4 is representative of samples with any greater percentage of overtly vacuolated cells.

Gonad maturity was assessed on a scale of 1 to 4 based on the percentage of cells from each stage of gametogensis, E.g. spermatogonia, spermatocytes, spermatids, and spermatozoa in male samples. In this regard a 1 represents 100% of visualized cells were

in the spermatogonia stage and a 4 represents 100% of the cells had achieved the spermatozoa stage. The end formula for this calculation would then be visualized as:

$$\text{Gonad maturity rating} = ((\%\ \text{spermatogonia}) + (\%\ \text{spermatids} \times 2) + (\%\ \text{spermatids} \times 3) + (\%\ \text{spermatozoa} \times 4))$$

Kidney and gill tissues were analyzed on an informal basis in an attempt to elucidate the existence and extent of respective anatomical pathologies identified in previous studies (Hoeger et al, 2005, Mehinto, Hill, & Tyler, 2010, Schwaiger et al, 2004).

Hematological Endpoints

BGLs recordings from caudal vasculature blood were attained via a hand-held digital TRUEbalance Blood Glucose Monitor (Moore Medical, Farmington, CT). Additional blood was collected in plastic heparin-coated capillary tubes from the same caudal vasculature and spun in a HERMLE Z200A centrifuge (Labnet International Inc., Woodbridge, NJ) at 4000 rpm for 3 minutes. Hematocrit was recorded using a Spiracrit Micro-Hematocrit Tube Reader (Clay-Adams Inc., New York, NY) prior to plasma being isolated and stored in 2.0 mL plastic centrifuge tubes at -40°C.

Cortisol concentrations from plasma samples collected on days 18 and 31 were assessed using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI). Due to a disparity between the quantity of blood plasma called for by the manufacturer instruction manual (50 µL) and the low volumes retrieved from each specimen (typically <10 µL) modified published vitellogenin ELISA procedures were utilized (Feifarek, 2015).

Statistical Analysis

All endpoints were analyzed using an ordinary one-way analysis of variance (ANOVA) and were subjected to a Dunnett's post-test. Normality was assessed using both Brown-Forsythe test and Bartlett's test. All statistical analysis was performed using Graphpad Prism software (Prism 5.0 statistical package, Graphpad Software, Inc, Oxnard, CA). In the interest of simplicity, results below are shown as "by day 18", or "by day 31", although half of the treatment groups were harvested within the two previous days for each daily increment. E.g. while fish from the methocarbamol, mix-all, and temazepam treatments were dissected on experimental days 5, 16, and 30, results for these groups are displayed alongside those of diclofenac, ethanol control, and sulfamethoxazole treatments which were dissected on days 7, 18, and 31.

RESULTS

Mortality

No cases of mortality were observed in any of the treatment groups during the course of this experiment.

Morphometric and Organosomatic Indices

No significant variation ($p < 0.05$) was found in the body condition factor, hepatosomatic index, or gonadosomatic index endpoints between treatment groups and the EtOH control group by day 18 or by day 31 (Fig. 3.1a, b, c).

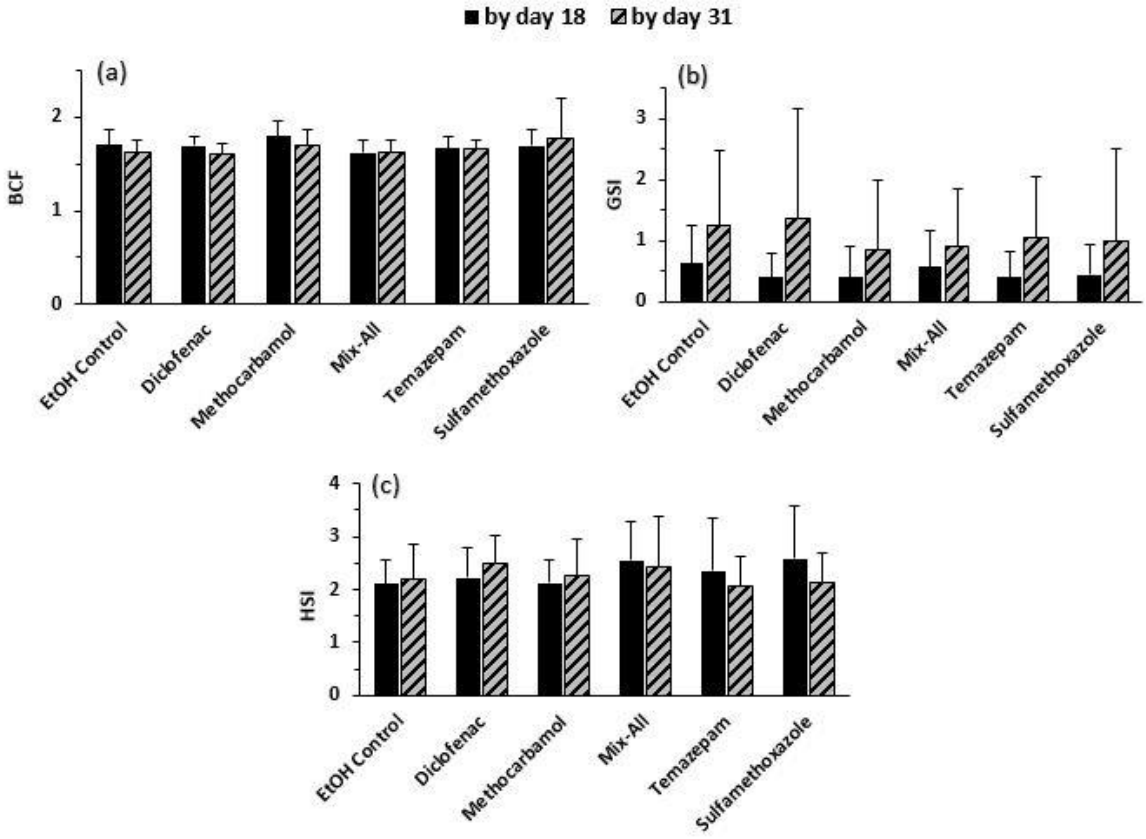

Figure 3.1. Mean morphometric (BCF; a) and organosomatic (GSI and HSI; b, c) indices of *L. macrochirus* after 18 and 31 days of flow-through exposure; methocarbamol: 4000 ng/L, all other treatments 2000 ng/L; no significant difference between means; error bars indicate one standard deviation; one-way ANOVA; Brown-Forsythe and Bartlett's post-test for normality; Dunnett's post-test to compare means.

Histopathology

Liver hepatocyte vacuole prominence was significantly affected ($p < 0.05$) in both the methocarbamol and sulfamethoxazole treatment group means by day 18. However, by day 31 there were no significant variations in mean vacuolization grades (Fig. 3.2).

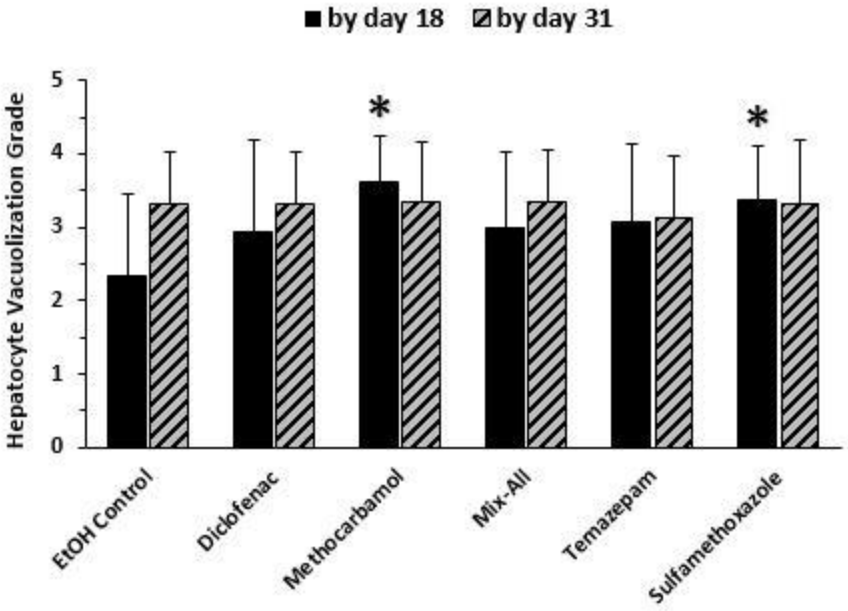

Figure 3.2. Mean hepatocyte vacuolization grades of *L. macrochirus* after 18 and 31 days of flow-through exposure; methocarbamol: 4000 ng/L, all other treatments 2000 ng/L; error bars indicate one standard deviation; asterisks above standard error bars indicate significantly different means ($p < 0.05$); one-way ANOVA; Brown-Forsythe and Bartlett's post-test for normality; Dunnett's post-test to compare means.

Mean gonad maturity rank was not influenced by different treatment groups by either the day 18 or day 31 dissection windows (Fig. 3.3).

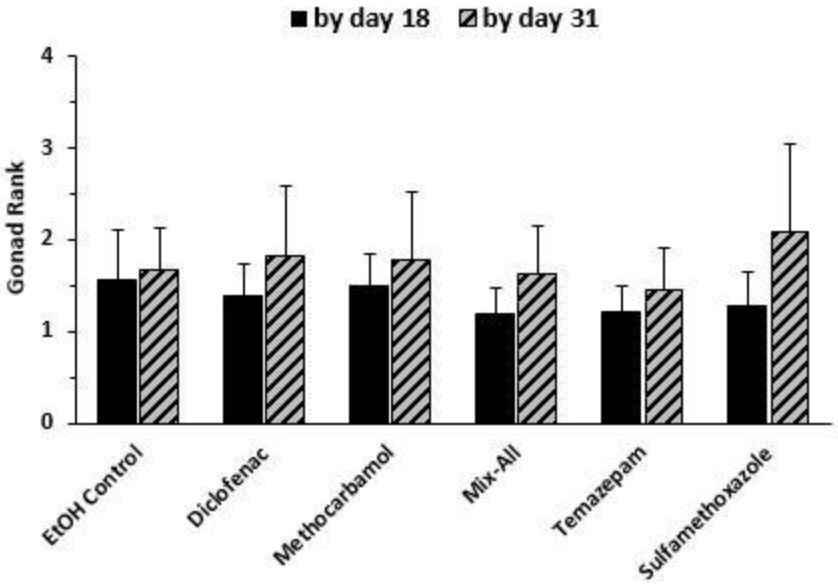

Figure 3.3. Mean gonad ranks of *L. macrochirus* after 18 and 31 days of flow-through exposure; methocarbamol: 4000 ng/L, all other treatments 2000 ng/L; no significant difference between means; error bars indicate one standard deviation; one-way ANOVA; Brown-Forsythe and Bartlett's post-test for normality; Dunnett's post-test to compare means.

A qualitative evaluation of kidney and gill histological samples from day 31 tissue dissections revealed the presence of several pathologies. Kidney slides showed evidence of loss of the Bowman's space in the renal corpuscle, while gill tissues revealed fusion of lamellae, interlamellar cell proliferation, lamellar clubbing, and respiratory epithelial cell necrosis.

Hematological Endpoints

No significant variation ($p < 0.05$) was found between means in BGLs or plasma cortisol concentrations (Fig. 3.4a, b). However, temazepam had a mean hematocrit by day 18 that was significantly lower ($p < 0.05$) than that of other treatment groups (Fig. 3.4c).

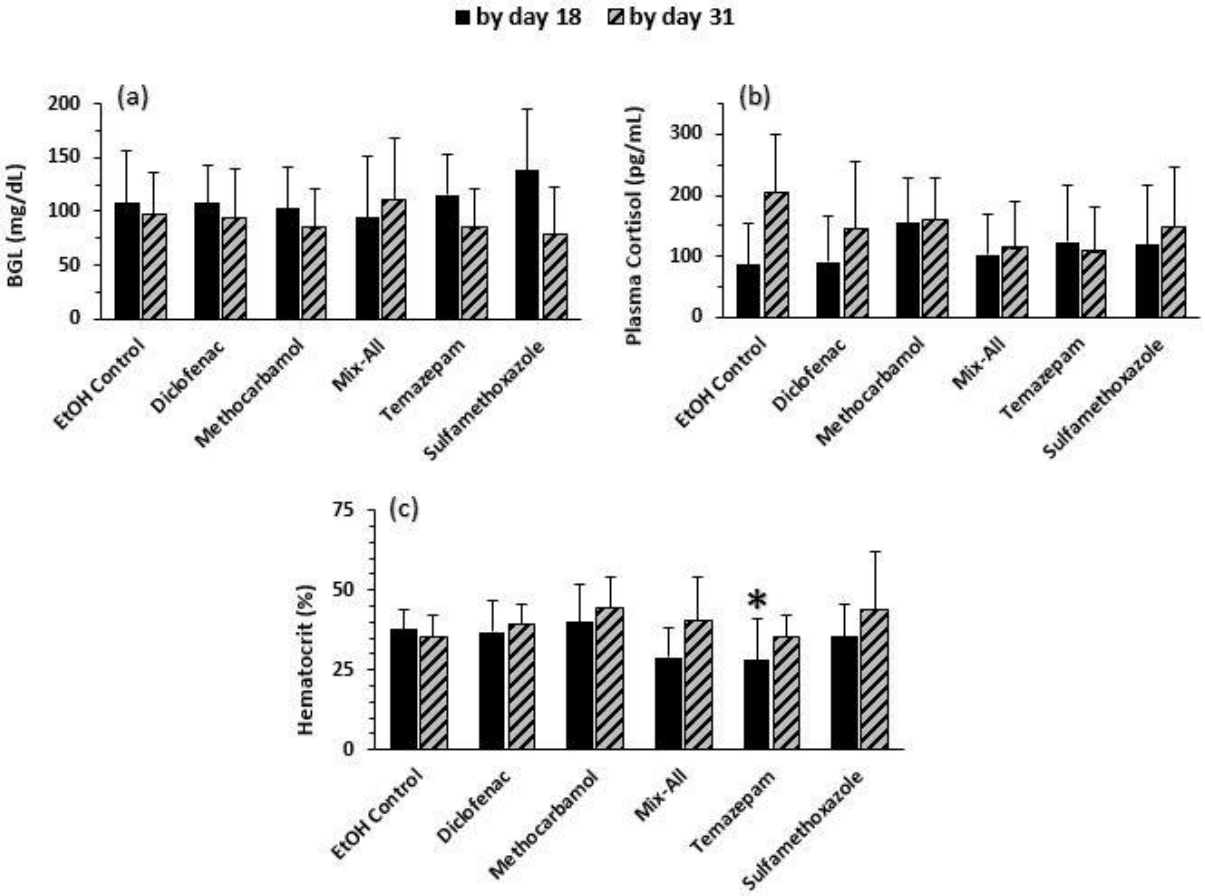

Figure 3.4. Mean blood glucose concentrations (a), plasma cortisol concentrations (b), and hematocrit (c) of *L. macrochirus* after 18 and 31 days of flow-through exposure; methocarbamol: 4000 ng/L, all other treatments 2000 ng/L; error bars indicate one standard deviation; asterisk above error bar indicates significantly different mean ($p < 0.05$); one-way ANOVA; Brown-Forsythe and Bartlett's post-test for normality; Dunnett's post-test to compare means.

DISCUSSION

The objective of this study was that of evaluating the potential for pharmaceutical contaminants of surface waters to induce adverse physiological and anatomical pathologies in bluegill sunfish. We predicted reduced overall health in individuals after 30 days of flow-through exposure as indicated by a depressed BCF and GSI, and elevated HSI. These predictions are based on the understanding that the fish exposed to contaminants would need to expend more energy metabolizing and removing the offending chemicals from their biological systems than the fish exposed only to ethanol. We also measured how disparities of this nature which are known to include manifestations of anatomical pathologies would become observable through histopathological techniques including hyper vacuolization of hepatocytes due to increased liver function demands and decreases in overall sexual maturity implicated by lower production of mature gametocytes. Additionally, and also in terms of signs relating to overall health, we expected to find variations in mean hematocrit, BGLs, and plasma cortisol concentrations which would further indicate elevated stress levels and physiological responses to the exogenous compounds. Data retrieved from fish exposed for the full 31 days of exposure do not support these predictions. However, it is worth noting that significant variation was found between select treatment groups and the control group after 18 days of exposure.

Despite the lack of significant findings in terms of BCF, GSI, and HSI, there are several aspects of these endpoints that are noteworthy. First is the lack of variability in BCF, a metric relating to fish health under the assumption that a greater body mass relative to length is indicative of better health, as the extra mass reflects stores of energy

otherwise depleted in less healthy individuals (Fulton, 1904, The Grayling Research Trust, n.d.). Such an endpoint is certainly valuable in obtaining information pertinent to environmental factors, yet may not provide great insight when variables are extremely limited as is the case in the above exposure experiments where all subjects were provided equal living conditions and more-than-adequate access to food sources. With this in mind we feel that the homogeneity of our BCF data set may simply be a combined veiling effect of the abundance of food and lack of competition or predation that would be found in the natural world. This is certainly not to insinuate BCF evaluation is without merit in all circumstances but rather that variables which would normally lead to significant impact in this regard may be readily susceptible to being covered up in the desire to limit discrepancies between treatment groups. We feel that this proposition is aptly extended to both GSI and HSI metrics as well, considering they are also inherently reliant on and intrinsic to a multitude of interconnected environmental factors in addition to those provided by a researcher in a controlled setting.

Perhaps the most surprising results were those pertaining to hepatocyte vacuolization, in which the only significant variation found was by day 18 for methocarbamol; a muscle relaxer, and for sulfamethoxazole; a sulfonamide antibiotic (Fig.3.2). Excessive vacuolization of hepatocytes is thought to be a response to acute liver injury (Hall et al, 2012, Nayak et al, 1996), in this case the result of prolonged exposure to pharmaceuticals. Such modification of hepatocytes involves recruitment of glycogen stores and has been shown to serve as a protective mechanism against toxins (Nayak et al, 1996). Our initial expectations were that we would note a continued exacerbation of liver cell modification throughout the course of the experiment.

However, the lack of significant variation may be explained by the length of the exposure itself, as vacuolization concentrations may have peaked prior to the glycogen stores being required for metabolism in the exposed fish. This would ultimately raise the question of whether, in the event of exposures lasting beyond the scope of our study, hepatocytes may begin to exhibit markers of cellular degeneration after losing the protective effects of their glycogen stores. A second hypothesis is that the treatment groups may have simply developed a modified physiological response to the pharmaceuticals resulting in some degree of resistance to our expected outcome. This thought process reinforces the need for continued and more in-depth investigation of liver damage resulting from prolonged exposure in future studies (Hoeger et al, 2005, Nayak et al, 1996).

Gonad rank measurements are targeted at evaluating the average sexual maturity of the treatment groups and ultimately are thought to be a reflection of how energy stores are utilized, where fish experiencing less stress or physiological demands are more likely to spend a greater amount of their metabolic resources on advancing their sexual maturity (Environmental Protection Department, 2005, Zeyle, Love, & Higgs, 2014). Reasoning of the same vein thereby insinuates an increased likelihood of suppressed or delayed reproductive maturity due to increased stressors such as contaminants. Surprisingly, this was not the finding of our work. However, it is worth considering that the devaluation of reproductive system growth may be intrinsic to the availability, or lack thereof, of energy resources. We propose that in a research environment where food sources are abundant, the distribution of energy is likely to be even between both treatment groups and control groups as neither is truly in a position necessitating the triaging and prioritizing of metabolic needs. With this in mind gonad rank may be unable to truly reflect the

implications of pharmaceutical contaminants in field scenarios and ultimately underlines the need for evaluation of contaminant effects in environments outside of the laboratory.

Due to the intricacies of histological evaluation of kidney and gill tissues and the proficiency of the individuals performing histology techniques, these samples were only evaluated on a qualitative basis with the intent of identifying the presence of organ level pathologies. The goal of this histological surveying was that of creating an educated path for future studies. Findings were limited to those identified and published in previous research and subsequently validated in a pathology working group review (Hoeger et al, 2005, Mehinto, Hill, & Tyler, 2010, Schwaiger et al, 2004). While surveying day 31 kidney tissues we identified the presence of glomerular space reduction in multiple treatment groups, although were unable to find any evidence of hyaline droplet degeneration, interstitial nephritis or proteinaceous fluid, tubular necrosis, or disparities in developing nephron presence. In gill tissue there was overwhelming evidence for future investigation of pillar cell necrosis, lamellar clubbing, and thickened lamellar tips. However, it is worth noting that these findings were based solely on a limited number of tissues and were identified by an individual with limited histopathological training.

Hematological analysis elucidated some significant biological impacts of treatments on exposed fish. Past research has shown that variations in BGL, plasma cortisol, and hematocrit concentrations may be indicators of nutrient deficiency, increased stress levels, and hypoxia in fish (Heath, 1987, Iwama et al, 1999, Pottinger & Carrick, 1999). In our study we did not find any indication of significant variation in BGL or plasma cortisol concentrations despite fluctuations being present to some degree (Fig. 3.2a, b). Although BGLs failed to provide insight in the above research, it is our

position that it's continued utility as a biomarker in toxicological research is supported by the relative ease and feasibility of data collection in both laboratory and field settings. While a significant BGL deviation finding was expected, a lack thereof may be attributable to several aspects of our methodology, including proximity of feeding times to euthanasia and inconsistencies in recorded data due to the use of several different digital blood glucose monitors. The lack of significant changes in plasma cortisol concentrations is also surprising, as elevated cortisol production has been shown be a reflection of increased physiological stressors (Pottinger & Carrick, 1999). Interestingly though, we did see some degree of variation between treatment groups despite a lack of statistical significance, an indication that modifications to procedures in future studies may yield compelling evidence of variations in cortisol being modified by chemical exposure. Lastly, data pertaining to average hematocrit by day 18 did find a significant variation between the temazepam treatment group and the control group (Fig. 3.4c). Considering the known association between hematocrit and hypoxia (Heath, 1987), this finding may be due to an exposure induced modulation of metabolic function. It is worth noting though that significant findings were also present in the day 31 ANOVA data, however, the data contained evidence of violations of normality in the Dunnett's post-test. The reasoning for this finding may be multi-fold and we provide several hypotheses including an unusual degree of variability in our fish populations in terms of true hematocrit concentrations or individual responses to treatments, as well as the potential for measurement errors resulting from individual interpretation of capillary tube readings during data collection events.

Chapter IV: CONCLUSION

Key Findings

In conducting the preceding flow-through and static exchange exposure experiments we attempted to obtain information pertaining to the "biological effect" portion of ongoing investigations of surface water pharmaceutical contaminants. In summary, we performed a series of tests which evaluated how such contaminants may induce adverse effects at different life stages of susceptible organisms including behavioral modification in larval *Pimephales promelas* as well as morphological and physiological effects in adult *Lepomis macrochirus*. We found that exposure impacted feeding efficiency in the former and hepatocyte vacuolization and hematocrit were affected in the latter. It is our position that this research sets a precedent for the need for continuation and more in-depth evaluation of pharmaceutical contaminants in the future.

In the larval fathead minnow exposures, we observed significant mortality rates in many of the ethanol control treatment jars. The resulting lack of control group population size in both the feeding efficiency and artificial predation stimulus assays (Fig. 2.2, Fig. 2.3) is a source of concern for the true reflection of experimental outcomes in our statistical analyses. Given our expectations and the history of positive findings in similar studies (McGee et al, 2009, Painter et al, 2009) it is imperative that future research methodology incorporates measures to address this issue. Removal of unwanted aquatic organisms such as fungal colonies could conceivably be accomplished utilizing siphoning or filtration of the jar contents, although care would need to be taken to minimalize inconsistencies and handling artifacts. However, the finding that larval fathead exposure to 2000 ng/L of diclofenac may be responsible for inhibited feeding efficiency is cause

for concern and validates a need for replication in future studies, especially given the ubiquity of NSAID use throughout the world in both human and animal populations as well as known incidences of unintended exposure consequences (Green et al, 2004).

The effects of all pharmaceuticals in the bluegill flow-through experiments were significant only in data attained from individuals after 18 days of continuous exposure (Fig. 3.2, Fig. 3.4). More specifically, our data supports the conclusion that exposure to methocarbamol or sulfamethoxazole at environmentally relevant concentrations induces significantly greater hepatocyte vacuolization, and exposure to temazepam is complicit in significantly reduced hematocrit concentrations. Additionally, an informal histological evaluation of kidney and gill tissues harvested at day 31 was useful in identifying several anatomical pathologies that should be investigated in greater depth in future studies. Based on these results we feel that there is merit in additional studies, especially those with modifications to methodology such as an increased focus on the dynamics of morphological and physiological endpoints over a similar period of exposure.

Future Directions

It is important to note that the objective of this study was multi-fold and ultimately served a goal of setting an initial framework for future works. By utilizing the preceding methodology as guidance for such research, we believe that a significant amount of useful information may be attained to expand our understanding of the issue of surface water contaminants. There is no doubt that undertaking an experiment using live organisms is a complex initiative inherently riddled with variables that may affect the outcomes. With this in mind it is imperative that an honest evaluation of the work and

procedures is accomplished after completion to identify any areas of inconsistency and clarify future steps necessary for correction.

The results of the preceding experiments, while not supportive of all predictions, are indicative of the complex dynamics in which exogenous compounds may affect aquatic organisms in the wild. In this regard we propose that our findings from day 18 data be utilized to guide the timeline of future studies as well as dictate the frequency in which evaluations of endpoints are taken. Considering the complexities involved in physiological responses to environmental conditions, it is conceivable that measurements taken at increased frequencies may yield additional support to hypotheses relevant to the progression of pathologies.

Additionally, we believe that building on the preliminary evaluation of anatomical abnormalities we found in gill and kidney tissues in a histopathological method will serve to further elucidate the true extent of diseases caused by contaminants. This line of thinking revolves around the idea that progression of symptoms associated with acute insult to these organs will undoubtedly manifest in quantifiable ways. Providing a narrowed field of objectives should thus serve to make a cleaner process of investigation.

Lastly, the utility of laboratory environments in toxicology testing has undoubtedly aided in the overall goal of CEC research, yet we must not fail to address the inability of such work to truly represent the variables found in the field. It is our recommendation to future investigators that continued work on pharmaceutical contaminants is carried out in a way that incorporates both laboratory and field settings to address any concerns that may arise over inconsistencies between data sets from each.

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