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Developmental and Behavioral Responses of Early Life Stages of Fathead minnows (Pimephales promelas) to Urban Effluents Treated with Advanced-Oxidation Processes

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Amy Cavanaugh

An Abstract of a Thesis

In

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Submitted in Partial Fulfillment of the Requirements for the Degree of

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Abstract

Wastewater Treatment Plants (WWTP) are the main vector for pharmaceutical and personal care products (PPCPs) to enter waterways. Many PPCPs are lipophilic, allowing them to bioaccumulate and biomagnify within aquatic organisms. PPCPs have been known to alter fish behavior and physiological processes, such as nesting defense and sex ratios, and produce an increase in deformities. Advanced oxidation processes (AOPs) are novel ways for treating WWTPs effluents that use hydroxyl radicals to indiscriminately breakdown PPCPs. In this study, fathead minnows (*Pimephales promelas*) were used to examine the biological effects of AOP-treated municipal effluent as they developed from eggs to juveniles, in a 30-day life history test. During early life stages, there was a trend for heart rate to be lower in the Hydrogen peroxide/Ultraviolet light and WWTP Secondary effluent. Hatching success was not impacted. However, egg hatching occurred earlier in the Hydrogen peroxide/Ultraviolet light (H_2O_2), Peracetic Acid/Ultraviolet light (PAA), and Niagara River treatments compared to the Control, while eggs in the secondary effluent had a delayed hatching time. Larvae 12 and 13 days old were tested for predator avoidance using their C-start response, which was not different among treatments. At day 30, there was a significant difference in weight and length for fathead minnows raised in the H_2O_2 and PAA treatments which resulted in stunted growth at the juvenile stage. These results indicate that AOP-treated effluent can have an impact in the growth and development of larval fish near outflow areas and potentially affect their fitness in their adult life.

State University of New York
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A Thesis in Biology

By

Amy Cavanaugh

Submitted in Partial Fulfillment of the Requirements
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Introduction

Aquatic organisms are routinely exposed to wastes generated from human activities. However, we are still discovering to what extent this multitude of wastes impact aquatic life. Wastewater treatment plants (WWTP) serve the vital purpose of recycling urban wastewater back into aquatic environments. However, recent data has shown that modern WWTP techniques do not adequately account for the removal of some types of waste, including pharmaceuticals and personal care products (PPCPs) (Blair et al. 2013, Keen et al. 2016). PPCPs encompass a wide array of chemicals and include, but are not limited to, antidepressants, antibiotics, over-the-counter drugs, fragrances, and sunscreens. These contaminants enter WWTP by being poured down drains and through human excretion but are not removed by the current treatment process, and in turn are present in the effluent released (Murphey, Brown, and Gandhi, 2013).

Since the 1980's, PPCPs have been detected in surface waters worldwide (Waggot et al. 1981, Watts et al. 1984) and, nowadays, PPCPs are ubiquitous in surface waters and are typically found in parts-per-billion (μg/L) and parts-per-million (mg/L) concentrations (Blaire et al. 2013, Arnnok et al. 2017). Although these concentrations may seem insignificant, PPCPs are problematic because they are lipophilic and bioaccumulate within organisms, contributing to their xenobiotic load (Coogan et al. 2007). For example, in the Niagara River, the metabolites of antidepressants have been measured at concentrations between 0.1 and 1.4 parts per billion in surface water. These metabolites have been found to have bioaccumulation factors as high as 1,600 in the brains of 11 different fish species (Arnnok et al. 2017). The impact of PPCPs on aquatic organisms is further exacerbated by the fact that they are active molecules, as many are by-products of medications designed to interact with intracellular receptors at very low concentrations (Murphey et al. 2017).

The levels of PPCPs found in surface waters can impact the physiology, reproduction, and behavior of aquatic biota (Murphey et al. 2017). For example, *Daphnia magna* exposed to urban sewage effluent for multiple generations produced fewer males in simulated winter conditions (which is when they would produce haploid resting eggs that would hatch into males), indicating a greater susceptibility to population crashes (Baer et al. 2009). Male fathead minnows exposed to urban effluent had altered liver and testis gene expression and reduced nest defense behavior (Garcia-Reyero et al. 2011). Adult zebrafish (*Daniero rerio)* exposed to either a mix of pharmaceuticals or effluent, showed atretic oocytes (breakdown of ovarian follicles), altered ovarian histology, and apoptosis in granulosa cell layers (Galus et al. 2013). In this same study, zebrafish embryos exposed to both, pharmaceuticals and effluent, had increased deformities and mortality. These examples provide evidence that PPCPs are some of the most dangerous components of urban effluent and have similar deleterious effects as would a mix of active pharmaceuticals and toxic products. Improved removal of PPCPs from sewage effluent is a necessary step to decrease the physiological and behavioral problems that result from the presence of these contaminants in aquatic ecosystems.

Advanced Oxidation Processes

Advanced oxidation processes (AOPs) are treatment technologies utilize hydroxyl radicals (•OH) to degrade recalcitrant compounds. Recalcitrant compounds persist in receiving environments. Hydroxyl radicals are highly reactive because they readily steal hydrogen atoms from other molecules to form water and CO_2. AOPs react unselectively, are short-lived, and have the capacity to remove, inactivate, or transform a wide range of pollutants into secondary byproducts. Considering the wide range of pollutants entering WWTP, AOPs are a potentially

powerful tool for cleaner WWTP effluent (Gligorovski et al. 2015). However, because residual oxidants, resulting from the action of the AOPs on effluent, have the potential to form carcinogenic compounds, thiosulfates ($Na_2S_2O_3$) are added as a final step in the effluent cleansing to quench excess oxidants and transform these residual molecules into innocuous salts and water (Sichel et al. 2011).

An advantage of applying AOP to WWTP's effluent is that they are cost-effective as they can be implemented at the end of the wastewater treatment without overhauling the facility. However, AOPs vary in quality and price. The least expensive AOP is ultraviolet light (UV), added to the already existing chlorine disinfectant stage. The addition of UV light provides energy to induce the production of hydroxyl radicals (Kim et al. 2009, Luo et al. 2014). Though chlorine/UV is cost-effective it degrades very few PPCPs and produces toxic chlorine byproducts compared to hydrogen peroxide/UV and Peracetic Acid/UV (Kim et al. 2009, Rott et al. 2018, Zhang et al. 2019).

Though more expensive, H_2O_2/UV and peracetic acid (PAA)/UV are more effective AOPs and have been found to degrade up to 90% of contaminants in effluent (Kim et al. 2009, Wols et al. 2013, Cai et al. 2017). These two AOPs, PAA/UV and H_2O_2/UV, have the same disinfectant action. However, at equal amounts/doses PAA has been found to be significantly more effective than H_2O_2 (Lubello, Caretti, Gori, 2002). This difference may result from PAA/UV generating carbon-centered radicals (i.e. CH3C(=O) O•, CH3C(=O) O_2•) in addition to •OH radicals. Carbon-centered radicals have strong reactivity to certain naphthyl PPCPs such as naproxen (commercially sold as Aleve) and 2-naphtoxyacetic acid (common plant fertilizer) (Cai et al. 2017).

AOPs are a realistic solution to the growing concern of PPCPs in effluent. However, the additional costs of UV light and chemical oxidant (e.g., hydrogen peroxide, peracetic acid) are a potential impediment to implementation. Furthermore, while there is a growing body of knowledge regarding effectiveness of AOPs (Gligorvski et al. 2014, Luo et al. 2014), few studies have examined the biological impact that releasing AOP-treated effluent to a body of water will have on the aquatic biota in that ecosystem.

Fathead Minnow: Model Organism for Toxicology Bioassays

Fathead minnows (*Pimephales promelas*) belong to the cyprinid family and the adults measure between seven and ten centimeters. This small forage fish is widespread throughout North America and is native to the entire Great Lakes basin. Fathead minnows tolerate wide ranges of pH, alkalinity, turbidity, and temperature, and are able to produce eggs at any time in a laboratory setting. These characteristics have contributed to making fathead minnows the most widely used test organism in ecotoxicology (EPA 1987, FishBase: *Pimephales promelas*).

While the effects of AOP-treated effluents on aquatic biota have not been widely investigated, there is a rich history on the effects that urban-treated effluents containing environmentally relevant levels of contaminants have on the fathead minnow. These effluents affect the fathead minnows' morphology (Parrot et al, 2002, Kidd et al. 2007), general behavior (McGee et al. 2009, Painter et al. 2009, Weinberg et al. 2014), predator avoidance behavior (Painter et al. 2009, McGee et al. 2009), and overall condition (Sowers et al. 2009). However, the most susceptible stage of fish to environmental pollutants is the larval stage. Fathead minnow embryos hatch after five days and begin exogenous feeding two days post-hatch. The juvenile stage is reached 30 days post-hatch (Parrott et al. 2006). The embryo, larval and early juvenile

stages are more vulnerable than adults to chemicals in water, and this vulnerability can be used to determine the biological consequences of exposure that in adults may go unnoticed (McKim 1977). A 30-day early life-stage test can be used to determine lethal and sublethal effects of complex chemical mixtures, such as treated effluents. This test is typically performed with less than 24-hour-old fathead minnow embryos and measures the effects of chemicals on their health such as hatching success, weight, and gross morphological alterations (Ankley and Villenueve 2006, Organization for Economic Cooperation Development 2013).

A crucial factor for the survival of fathead minnows is their ability to avoid predators (Houde and Hoyt 1987, Batty & Domenici, 2000). Foraging movements by predators create stimuli (i.e., ripples as they swim) that are perceived by fish larvae. After a danger stimulus has been received, a reflex that helps larvae swim away from potential predators called the C-start response is a very common larval escape tactic (Eaton et al. 2001). The reflex begins with a short latency period during which a stimulus is perceived, followed by a dramatic bending of the body into a C-shape, and ending with an explosive burst of high-velocity swimming away from the predator stimulus (Domenici and Batty 1997). If this behavior is delayed, the predator avoidance performance will be insufficient, and possibly reduce the survival chances of the fish larva.

The physiological response of the C-start behavior, as a result of a predator stimulus, is controlled by an integrated sensory-motor axis. In the hindbrain, reticulospinal neurons MiD2 cm and MiD3 cm, assist specialized neurons known as Mauthner cells to activate a musculo-skeletal response to the stimulus (Faber et al. 1991, Eaton et al. 2001). Toxicants can impair neurotransmitters by altering presynaptic neurotransmitter production, binding neurotransmitters, and/or blocking postsynaptic receptors. Any of these alterations can disrupt communication between the afferent sensory receptors and the efferent motor system and result in reduced

behavioral performance (Little and Brewer 2001). Furthermore, any morphological or muscular deformities could also impair behavioral performance. Several studies have shown that WWTP effluents can impact the predator avoidance performance of fathead minnows (McGee et al. 2009, Painter et. al 2009). Therefore, C-start assays can provide quantifiable data to assess how PPCPs may impact the health and fitness of fish larvae (McGee et al. 2009, Painter et al. 2009).

Objective

The objective of this study is to assess and evaluate the effects of WWTP-effluent treated with advanced oxidation processes (AOPs) on the early life stages of fathead minnows (from egg to juvenile). Specifically, this project focused on their survival, development and behavioral responses.

In this study, the experimental treatments used were:

- Peracetic Acid 6 mg/L + UV 3000 mJ/cm^2
- Hydrogen Peroxide 6 mg/L + UV 3000 mJ/cm^2
- Secondary WWTP effluent, not AOP treated
- Niagara River Water
- Control (dechlorinated and filtered municipal tap water)

Hypotheses

Hypothesis 1: Fathead minnow embryos raised in AOP-treated effluent would be expected to have lower heartbeat rate, fewer morphological abnormalities and hatch at a greater rate than fathead minnows raised in secondary effluent or Niagara River water.

PPCPs have been shown to cause increased heartbeat, developmental abnormalities such as pericardial edema, hemorrhage, and curvature of the spine (He et al. 2012, Kingcade et al.,

2021). Also, municipal wastewater effluent has been shown to cause developmental delays that result in delayed hatching (Gauthier and Vijayan, 2020). Considering H_2O_2/UV and PAA/UV break down as much as 90% of PPCPs in municipal effluent (Kim et al. 2009, Wols et al., 2013), I am expecting fish raised in these AOP treatments to have fewer abnormalities and a greater hatching rate. Considering PAA/UV has been found to be more effective than H_2O_2 at equal doses, I expect fathead minnows raised in PAA/UV to be the most similar to the Control (Lubello, Caretti, Gori, 2002).

Hypothesis 2: Fathead minnow larvae raised in AOP-treated effluent would be expected to have a faster C-start response compared to larvae raised in secondary effluent or Niagara River water.

The C-start response has been found to be delayed when fish are raised in treatments containing antidepressants (Painter et al. 2009) and estrogen compounds such as those found in birth control pills (McGee et al. 20009). Considering that AOP-treatments are effective at breaking down PPCPs, I expect that larvae raised in PAA/UV and H_2O_2/UV will have a faster response than larvae raised in the secondary effluent or in the Niagara River water.

Hypothesis 3: Fathead minnow juveniles raised in secondary treated effluent would be expected to have lengths, weights, and condition factors similar to juveniles raised in AOP-treated effluent. Thus, the AOP-treated effluent should not affect larval growth.

Fathead minnow raised in effluent compared to groundwater for 30 days had equivalent weights (Barber et al. 2007). Similarly, when fathead minnows were exposed to WWTP effluent for two generations, Sowers et al. (2009) reported similar weight, length, and condition in exposed fish and the Control group.

Methods

2.1 Effluent Collection and AOP Treatments

For this study, effluent was collected and treated at Dr. Ning's Lab (University at Buffalo). Effluent was collected at an Amherst, NY, WWTP on January 8th, 2020, from the secondary clarifier. In the lab, advanced oxidation processes (AOPs) were carried out in a batch reactor. All effluent treatments were filtered with a 7 µm glass-fiber filter (pre-combusted). In 2 L batches using a beaker, oxidants (i.e. peracetic acid, hydrogen peroxide) were added and the beaker irradiated with UV light. Next, thiosulfate solution (6 g/L $Na_2S_2O_3$) was added to the beaker to quench excess oxidants. To evaluate the remaining PPCP concentrations after the AOPs' treatments, experimental fluids were analyzed using a mass spectromer (Figure 1).

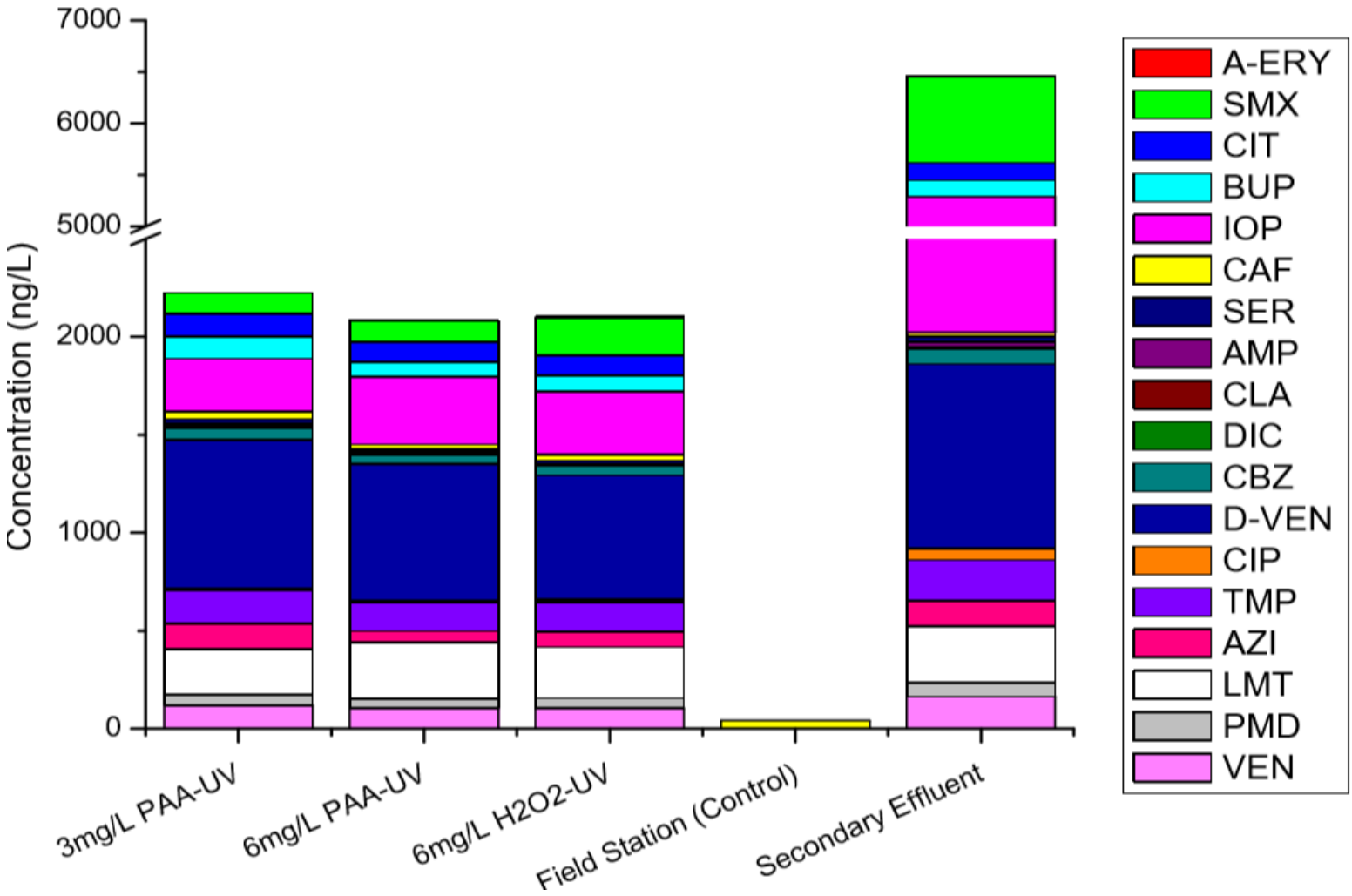

Figure 1. Mass spectrometer analysis of treatments for PPCP presence. Concentration (ng/L) of 18 targeted PPCPs. The Field Station Control reflects Buffalo's tap water. The abbreviations are A-ERY: Anhydro-Erythromycin, SMX: Sulfamethoxazole, CIT: Citalopram, BUP: Bupropion, IOP: Iopamidol, CAF: Caffeine, SER: Sertraline, AMP: Amitriptyline, CLA: Clarithromycin, DIC: Diclofenac, CBZ: Carbamazepine, D-VEN: Desvenlafaxine, CIP: Ciprofloxacin, TMP: Trimethoprim, AZI: Azithromycin, LMT: Lamotrigine, PMD: Primidone, VEN: Venlafaxine. The PPCPs categories and values are listed in Appendix A, Table 1.

In this test of AOP efficiency at removing pharmaceuticals (Figure 1), it is clear that all the different AOPs tested removed significant amounts of PPCPs (compared to the secondary effluent), but not all (compared to the Control). The most prevalent PPCP in the effluent, Iopamidol, was also the most reduced by the AOP treatments. Iopamidol is a contrast agent used for computerized tomography (CT) scans and other radiological examinations. The Field Station (Control) water contained trace amounts of caffeine. Therefore, the Control water used for the fathead minnow bioassays was filtered for removal of impurities.

Experimental Treatments for Fathead Minnow Bioassays

In this experiment, I used five treatments. Two treatments were effluents treated with AOPs (with thiosulfates added to quench excess hydroxyl radicals): 6 mg/L peracetic acid with exposure to UV light at 3000 mJ/cm^2 and 6 mg/L hydrogen peroxide with exposure to UV light at 3000 mJ/cm^2. Three treatments were non-AOPs: Secondary effluent without any additions, Niagara River water, and a Control of filtered Buffalo municipal tap water. The Buffalo tap water was sourced from Lake Erie and was filtered through a Carbon filter and a Redox KDF filter to eliminate impurities and residual pollutants. The Niagara River water was collected on October 4th, 2020, about two hundred feet north of the Sheridan boat launch in Tonawanda, NY. The Niagara River water was then filtered through Whatman filter paper (cat No. 1001 055) to remove sediment and live biota. All treatments solutions were frozen at -80^{o}C for later use, at which point they were thawed with a water-bath and kept at 20°C. Bioassays were performed using the five treatments listed in Table 1 and will be referred to by the abbreviations.

Table 1: Treatments used for fathead minnow bioassays.

Treatment	Abbreviation
6 mg/L peracetic acid + 3000 mJ/cm^2 UV light, *AOP treatment	PAA
6 mg/L hydrogen peroxide + at 3000 mJ/cm^2 UV light, *AOP treatment	H_2O_2
Secondary effluent without any AOP treatments	Secondary effluent
Niagara River water	Niagara
Municipal tap water, ran through a redox/carbon filter	Control

2.2 Fathead Minnow Breeding

To examine the effects of AOP-treated effluent on vertebrates, fathead minnows were raised in the experimental water treatments from egg to juvenile. Fathead minnows were bred at the Great Lakes Field Station to obtain eggs < 24 hours old for the bioassays. Parent fish were purchased from Whispering Pines Fish Farm in Holland, NY. Whispering Pines is a Department of Environmental Conservation approved hatchery, and all fish were raised in outdoor ponds fed by spring water. Adult fathead minnows were maintained in 75 L tanks. To induce secondary sexual characters, I placed spawning tiles (10.8 cm clay flowerpots cut in half) in the tanks. A photoperiod of 16 h light: 8 h dark and water temperature of 24 ± 1ºC were maintained to simulate summer conditions that promote spawning activity. Fish were fed twice daily: frozen brine shrimp (*Artemia spp.)* in the morning, and frozen bloodworms (*Glycera spp.)* in the afternoon (Buttner and Duda 1986, U.S. EPA 2016).

Sexually mature fathead males display dark horizontal stripes on their body and raised tubercles around their snout. Sexually mature females are typically smaller in size and swell in their stomach area when gravid with eggs (Figure 2). Fish displaying sexual dimorphic characteristics were isolated by sex and moved into two separate 18 L tanks—one for males, one for females. For fish isolated by sex, photoperiod, temperature, and diet were maintained, but spawning tiles were removed to pause breeding behaviors. After seven days, fish from both sexes were reintroduced in breeding tanks, in a ratio of two females to one male (Buttner and Duda 1986, U.S. EPA 2016).

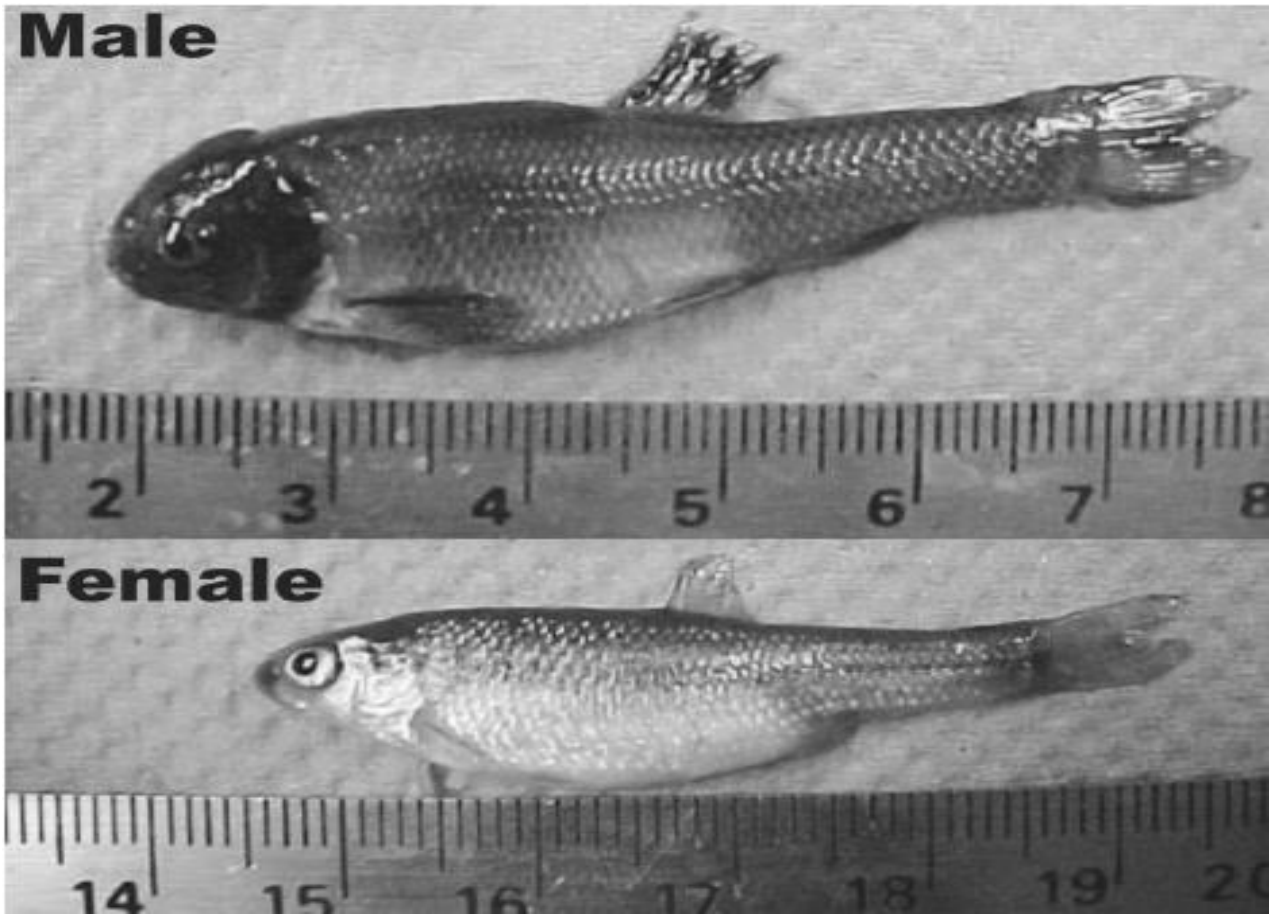

Figure 2. Mature male fathead minnow (top), mature female fathead minnow (bottom) (Parrot 2005).

Breeding tanks were 75 L and divided in two halves widthwise which allowed for two separate breeding groups per tank. The halves were separated with an opaque screen (food-grade plastic, Dexas Dx Grippmats) that was cut to fit the tank and held in place with suction cups. Holes in the screen allowed water to flow between sides (Figure 3). Photoperiod, temperature, and diet were maintained.

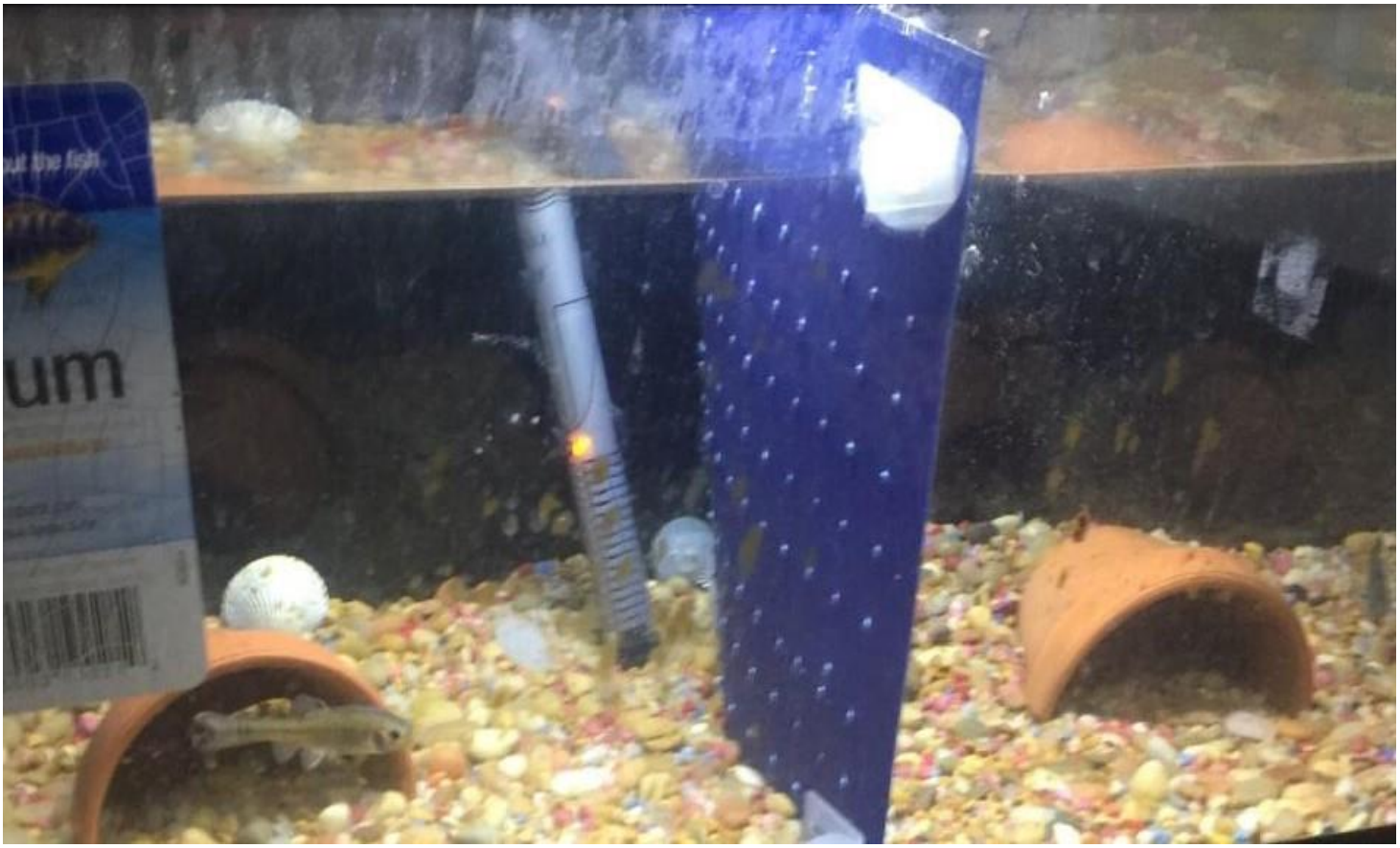

Figure 3. Fathead minnow breeding tanks consisting of one large tank, with an opaque divider. Breeding tiles were clay pots cut in half. Fathead minnows were bred in Fall 2020 at the Great Lakes Field Station (Buffalo, NY).

Each morning breeding tiles were checked between 10:00 a.m. and 11:00 a.m., as spawning primarily occurs with the onset of light. Upon spawning, egg covered tiles were transported to Buffalo State's campus in a beaker filled with water from the breeding tank. Upon arrival, an aerator tube was added to the beaker, and the beaker was placed in an incubator set at 16 h light:8 h dark and 23°C (Buttner and Duda 1986, U.S. EPA 2016).

2.3 Eggs with Developing Embryos: Experimental Set-up

www.All eggs used for the bioassays were < 24 hours old. Eighty mL of each treatment, and an additional 1 L of Control water were warmed to 23ºC in a water bath. Eggs were removed by finger rolling and placed in a 1 L beaker of Control water. This beaker was aerated for five minutes to ensure eggs were well mixed. All eggs were placed in a communal Petri dish filled with Control water and assessed for fertilization with a dissection scope. Unfertilized or abnormal eggs were discarded. Remaining eggs were then stirred with a glass rod to ensure randomization, and then distributed into Petri dishes filled with the corresponding treatment water. To ensure against any dilution from the communal Control-water filled Petri dish during dispersion, eggs were transferred again into a Petri dish filled with the corresponding treatment (Parrott et al. 2002).

Next, eggs were transferred from treatment-filled Petri dishes into 96-well polystyrene plates (Falcon® 96-well Clear Flat Bottom) that had been soaked in control water for 24 hours to remove potential polystyrene leachates. Each egg was moved using a pipette with 250µL of treatment water. Eggs were placed in every other well to ensure their identity if they accidentally were moved. Each treatment had two plates (18 eggs per plate, n= 36 eggs per treatment). Lids were placed on well-plates, and then plates were randomly distributed in the incubator in two

rows of five. The randomization was determined with a random list generator (https://www.random.org/). The incubator was set to 23º C, 16 h light:8 h dark photoperiod. Complete water changes across all treatments were done daily by drawing up the treatment water with a pipette, and then immediately refilling with fresh, aerated treatment water. Plates were then placed back in the incubator in a new randomized order.

Heartbeat Scores, Days 1-4

Embryo heartbeats have been shown to be an early indicator of toxicity (He et al. 2012). The scores for embryo heartbeats were obtained after randomizing the eggs at two levels: plate and egg order within wells. Plate and embryo orders were changed daily.

To count heartbeats, well-plates were placed under a dissecting scope, which had been fitted with a red covering over the light source to decrease perturbation of the eggs. Eggs were rested for 15 seconds to account for any perturbation from moving the well into the microscope's field-of-view. Headphones connected to a timer were used to ensure that the clock alert did not disturb the eggs. Heartbeats were counted with a mechanical clicker for 15 seconds. This number was multiplied by four to obtain heartbeats per minute. After heartbeats were measured for all eggs on a plate, the plate was placed back in the incubator.

Calculation of Hatching Rate, Day 5

Hatching success and hatching rate are indicators of fish fitness and were assessed for each egg. I determined hatching rate by the number of eggs that had hatched on day five compared to the total number of eggs. Day five was chosen because this was the day the majority of the eggs had hatched in the Control (81.2%). A binomial test was used as hatching only had

two possible outcomes (hatched/not hatched) and determined if hatching rate between treatments was significantly different than the Control. For the binomial test (Eq. 1), **C** was the combinatorial function, **n** was total number of eggs, **x** was total number of eggs that hatched in each treatment, and **p** was the expected hatching rate determined by the Control.

(Equation 1) $$C(x) = \left(\frac{n!}{x!(n-x)!}\right) * p^{x} * (1-p)^{n-x}$$

2.4 Larval Bioassays Set-up

Upon hatching, larvae were assessed for abnormalities using a 1 to 4 scale (1= no abnormality, 2= slightly abnormal, 3= moderately abnormal, 4 = highly abnormal, see example in Figure 4). Fish that received an abnormality score > 1 were photographed with a high-resolution Olympus DT 21 microscope digital camera, and the abnormality was described following He et al. (2012).

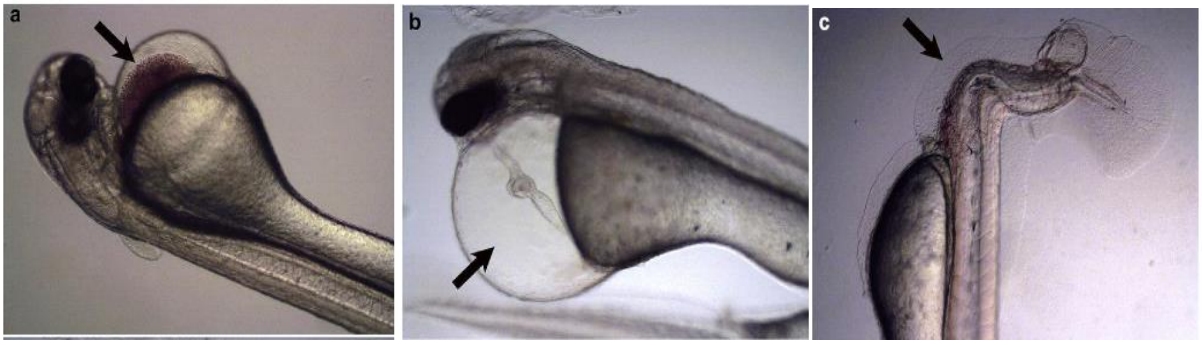

Figure 4. Photographs of abnormal fathead minnow larvae (a) hemorrhage, (b) pericardial edema and (c) malformation of the spine (20X magnification) (photographs from He et al. 2012).

For this second phase of the experiment, the larvae that hatched on days five and six were placed in a beaker of their same water treatment. The minimum number of fish that hatched in a treatment that could be distributed evenly amongst eight beakers determined the number of larvae for the next phase of the experiment. Thirty-two larvae was the minimum number that hatched in a given treatment, thus 32 larvae were randomly distributed between eight beakers for each treatment. The result was eight beakers per treatment, each containing four fish (n = 32 per treatment). Each day, 50% static renewal water changes were completed using aerated treatment water. Any dead larvae were removed and frozen at -80ºC. After water changes, beakers were randomly distributed in the incubator in eight rows of five. Order was determined with a random list generator. The incubator remained at 23 ± 0.4ºC, 16 h light: 8 h dark.

It has been established that fathead minnow larvae should be fed at least 150 brine shrimp (*Artemia spp.)* nauplii per day (Environment Canada 2011). To ensure equal feeding across all larvae and reduced food competition, larvae were fed *ad libitum* (> 200 nauplii/larva/day). Newborn nauplii were fed daily to the larvae to account for their small mouth gape. To grow the brine shrimp, a 500 mL separatory funnel was filled with 35 ppt salt-water solution. Brine shrimp cysts were added in a ratio of 0.5 grams/500 mL of salt water. An aerator was placed at the bottom of the funnel to prevent cysts from clumping together. After 24 hours, nauplii were drawn into a 20 µm-mesh filter and rinsed with deionized water.

To determine brine shrimp density, petroleum jelly was smeared on a microscope slide and 0.01 mL of well-mixed brine shrimp nauplii solution was transferred onto the jelly, fitted with a slide cover, and counted to calculate the appropriate amount of brine shrimp solution to feed the fish. At day six, food was increased to 300 nauplii/larva/day to account for increased body size (U.S. EPA 2016).

2.5 Evaluation of the C-Start Response in Larvae 12 and 13 Days Old

An important factor in the survival of fathead minnows is their ability to avoid predators. The C-Start response assay was used to test the effects the different treatments had on predator avoidance, and the methods were adapted from Bird (2015). A nine-centimeter plastic Petri dish was fitted onto a light box. The dish was filled with 15 mL of room temperature Control water and placed on a 1 mm grid. This grid allowed for larval movements to be quantified. Four 10 x 3 mm vibrational disks were adhered to the bottom of the Petri dish in a square pattern for balance (Figure 5). One of these disks was connected to an electrical circuit board (Elenco Snap Circuits Beginner Electronics Exploration Kit, EE-SCB20), and emitted a 3V/12,000 RPM vibration when a trigger was pressed. This vibration simulates the motion waves of an approaching predator. A LED light that was shielded from the larva's view was also connected to the circuit board and activated simultaneously when the trigger was pressed. The LED light indicated time zero during the analysis of the recording sequences. A high-speed digital video camera (Casio Exilium, EX-FH20) capable of capturing 210 frames per second was placed 50 cm above the grid to capture the entire filming arena (Figure 6).

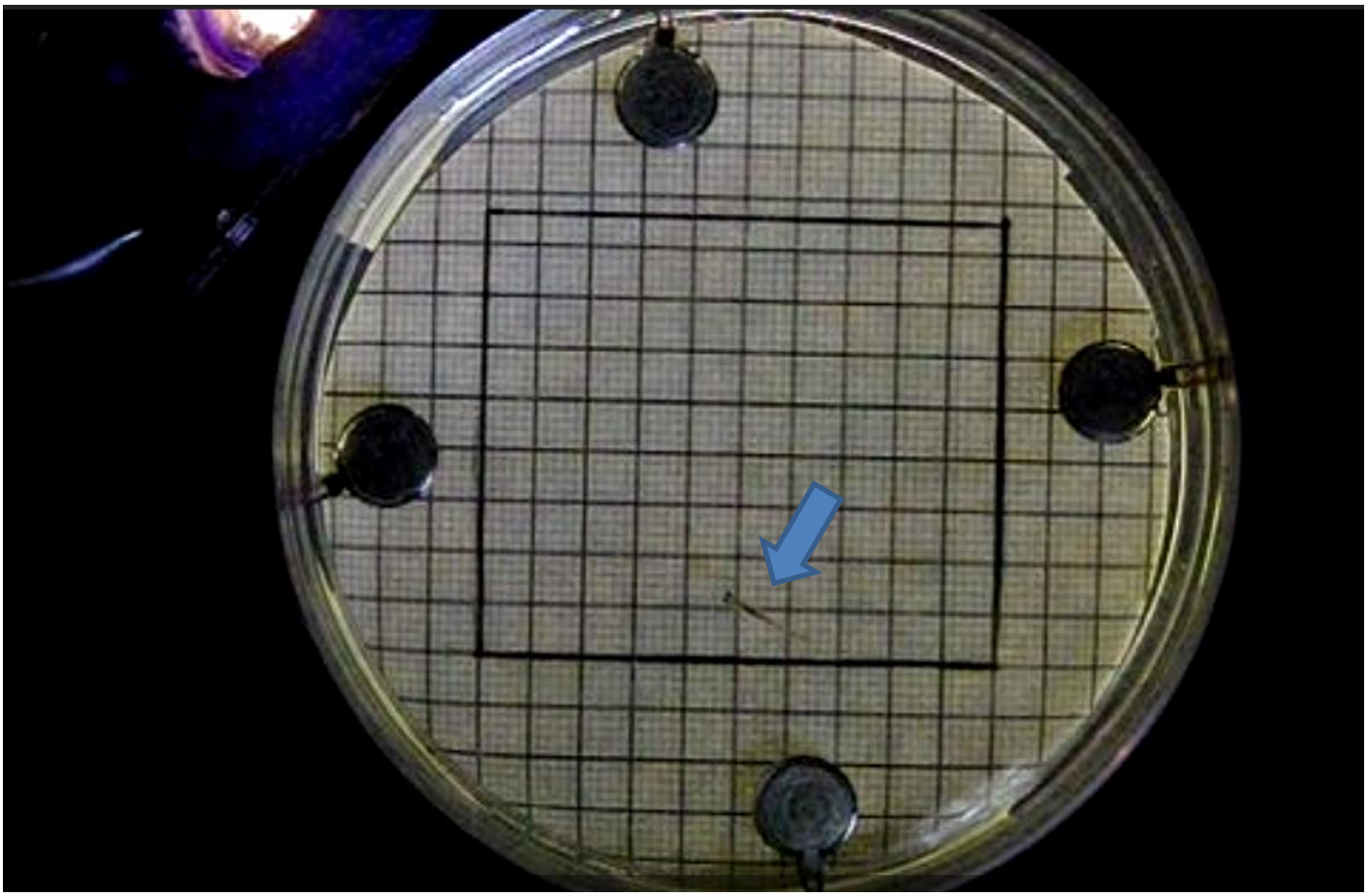

Figure 5. An aerial view of the C-Start arena used in this experiment to test predator avoidance response in 12 and 13 days-old larval fathead minnows. Arrow shows position of a fish larva.

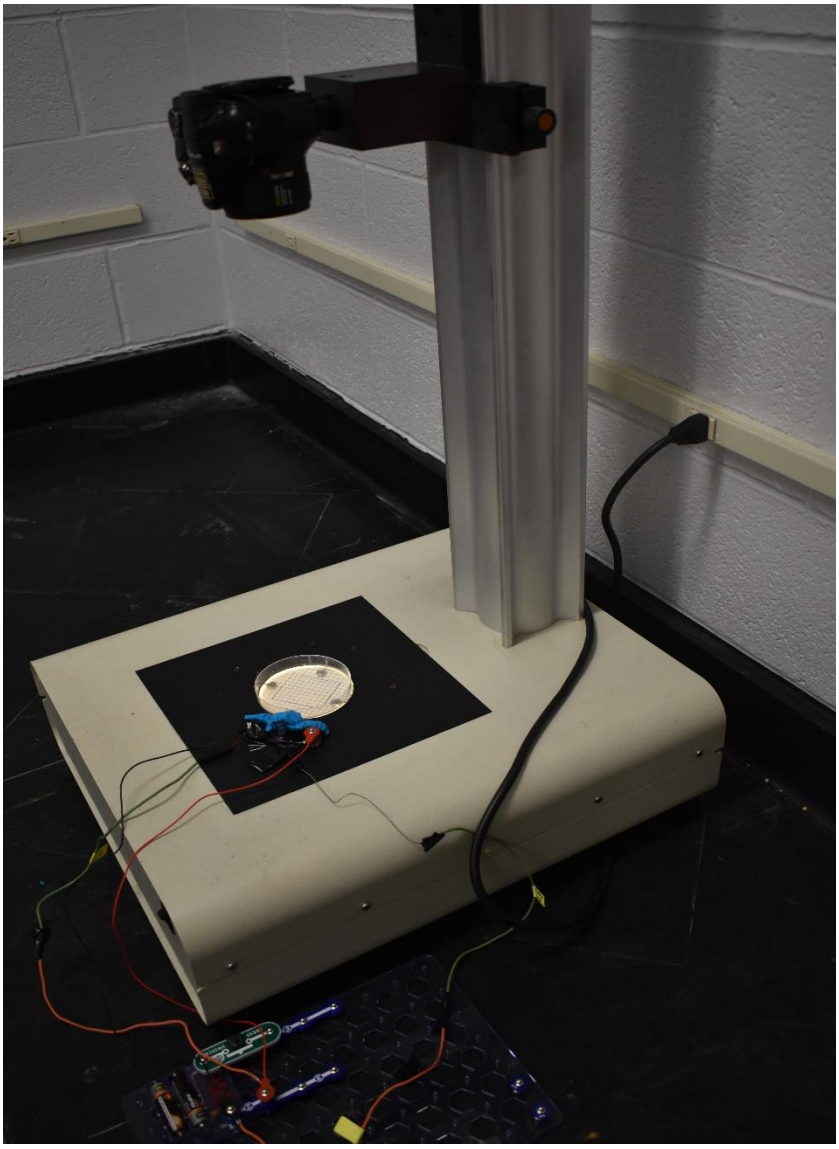

Figure 6. C-Start arena had a Casio Digital camera placed 50 cm above the stage to record the larval C-Start response.

Twenty minutes prior to testing, all larvae were fed brine shrimp nauplii *ad libitum* to avoid a drift response, also known as a slowed response due to time since last feeding as opposed to genuine differences. The test started after a larval fish was placed in the Petri dish, swam into the marked square at the center of the grid and paused (Figure 5). Once the larva paused in the

center square, the vibrational stimulus was activated and data on its behavior was recorded. After one exposure, the larva was removed, blotted dry and weighed to the nearest 10^{-2} mg, and frozen at -80°C.

Fish were tested individually in the order: Control, PAA, H_2O_2, Secondary effluent and Niagara, and then repeated, until sixteen fish per treatment were tested. The C-Start assay was carried out in three sessions of four hours on days 12 and 13. There was a minimum of two hours between each session.

The high-speed film recordings were saved as .AVI files and examined using ImageJ software (National Institute of Health). ImageJ was also used to determine length from the anterior tip of the head to the posterior tip of the tail for each larva. Length was taken at three separate points in the video recording of a given larva, and these measurements averaged.

The C-start assay had four endpoints*: latency period, escape velocity, escape angle,* and *total escape response*. *Latency period* was the number of milliseconds from the time the LED light turns on/vibration begins to the first head movement of the fish as it initiates a C-start response. *Escape velocity* was the distance traveled during the 42.857 ms after the first head movement (Eq. 2). *Total escape response* was calculated by combining *latency period* and *escape velocity* (Eq. 3). *Escape angle* was the deepest measured bend during the C-start response (Figure 7) (Painter et al. 2009, McGee et al. 2009, Bird 2015).

(Equation 2) *Escape Velocity* = $\left(\frac{\boldsymbol{Distance\ traveled\ (mm)}}{\boldsymbol{Body\ length\ of\ fish\ (mm)} \right)$ **/ 42.857 ms**

(Equation 3) *Total Escape Response* = $\left(\frac{\boldsymbol{Distance\ traveled\ (mm)}}{\boldsymbol{Body\ length\ of\ fish\ (mm)} \right)$ **/ 42.857 ms +** ***Latency)***

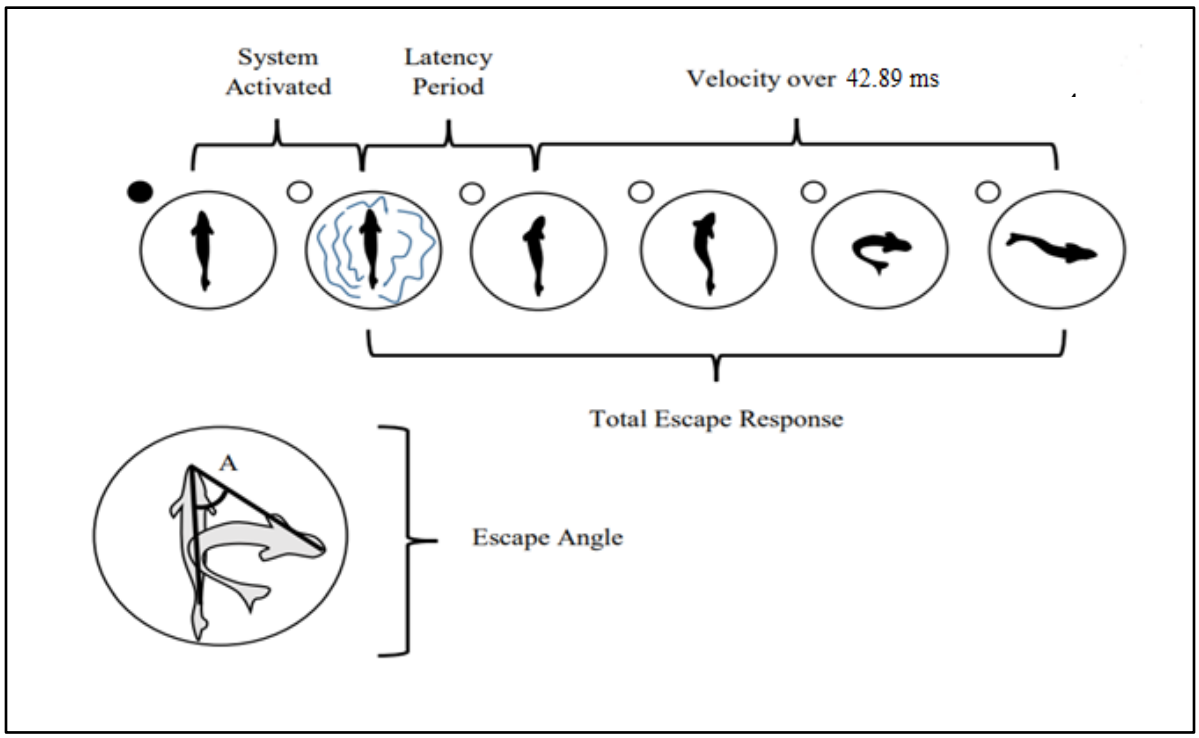

Figure 7. Schematic representation of predator avoidance behavior with the performance endpoints used in the C-Start Assay (Bird 2015).

For a video to be considered for analysis, the larva needed to (1) have a *latency response* of more than five milliseconds to ensure that it was reacting to the stimulus and (2) have its first response after the predator stimulus be an "escape turn" (Liu & Fetcho 1999). The exclusion of some recordings that did not meet these criteria resulted in some differences among sample sizes between treatments.

2.6 Relative Growth Pattern (b) and Condition Factor (K)

The experiment was terminated when larvae were 30 days old and considered juveniles (Propst and Stefferud 1994). To euthanize the juvenile fish, the individual beakers that contained the fish were placed in an ice bath until the fish stopped moving. Juveniles were then removed

with forceps, blotted dry, and wet weight was measured to the nearest 10^{-2} mg. Length was measured using an Olympus DP 21 digital camera. All fish were examined, and any abnormalities recorded. Fish were then frozen at -80º C (Parrott et al. 2002).

Fathead minnows display isometric growth, and juveniles take on the relative body shape of adults early in development (Propst and Stefferud 1994). Therefore, relative growth pattern, b, (Eq. 4) and Fulton's condition factor, K, (Eq. 5) are appropriate metrics for larval and 30-day-old fathead minnows.

(Equation 4) $$\mathbf{W = aL^b}$$

(Equation 5) $$\mathbf{K = \frac{W}{L\hat{}3}100}$$

The parameters for Eq. 4are obtained from the length-weight relationship power regression: **W** is whole body wet weight, **L** is total length, **a** is a parameter and **b** is an exponent that indicates isometric growth (≥ 3) or allometric growth (< 3). Fulton's condition factor parameters (Eq. 5) include: whole body wet weight (**W**) and total length (**L**). The factor 100 is used to bring **K** closer to unity. A larger **K** value indicates a rotund (fatter/thicker) body (Froese 2006).

2.7 Statistical Analysis

Heartbeats were not normally distributed (Shapiro-Wilk's test) and were analyzed using the non-parametric Kruskal-Wallis' test. The *post-hoc* analysis was carried out using Dunn's pairwise analysis with the Bonferroni correction ($p < 0.005$). Hatching rates were analyzed using a binomial test, with the expected hatching rate determined by the Control. Abnormality scores were analyzed with the non-parametric Kruskal-Wallis' test.

For the C-start endpoints, the Levene's test was used to assess homogeneity of variance, in which none of the models showed significance ($p > 0.05$). All parameters were tested for normality (Shapiro-Wilk's test) and log transformed when necessary. *Latency period, escape velocity, total escape response, and escape angle* were log transformed, and analyzed using ANOVA.

Larval weight and length were used to calculate Relative Growth Pattern (*b*) (Eq. 4) and Fulton's condition factor (Eq. 5). The Fulton's Condition Factor (log transformed), weight, and length were analyzed using ANOVA. *Post-hoc* analysis was completed using Tukey's Honest Significant Difference (Tukey's HSD). Principal Component Analysis (PCA) and a Pearson's Correlation Matrix was also used to further analyze *latency period, escape angle, velocity,* and Fulton's Condition Factor. *Total escape* is an equation derived from *escape velocity* and *latency period* (Eq. 3) and was not used in the PCA or Pearson matrix to prevent covariance.

The weights and lengths measured on day 30 were transformed and analyzed using ANOVA. Tukey's HSD was used for *post-hoc* analysis. Weights and lengths were also used to calculate *b* (Eq. 4) and Fulton's Condition Factor (Eq. 5). ANOVAs were used for Fulton's condition factor and *post-hoc* analysis was carried out using Tukey's HSD.

Results

3.1 Egg Stage and Hatching

Heartbeats

There were minor but significant differences in heartbeats per minute (heart rate) that varied between treatment and duration of time (Table 2). After being exposed to treatment for one day, heart rate in the Control was lower than the heart rate in the H_2O_2 and Secondary

effluent (Kruskal-Wallis, $p < 0.05$, Table 2, Figure 8A). By day three, the embryos raised in Secondary effluent had lower heartbeats than those in the other treatments (Kruskal-Wallis, $p < 0.05$, Table 2, Figure 8B). On day four, heartbeats were higher in the embryos raised in PAA than those in the H_2O_2 (Kruskal-Wallis, $p < 0.05$, Table 2, Figure 8C). Overall, there was a trend for heartbeats to increase over time, except for those treatments that were significantly different, such as the Secondary effluent on Day 3, and H_2O_2 treatment on Day 4. Across days, heartbeats were most similar in the Control and Niagara treatments (Figure 8D).

Table 2: Kruskal-Wallis results for heartbeats/minute of embryos across treatments. Measurements were taken on days 1, 3, and 4. Asterisks indicates significant difference ($p < 0.05$).

Kruskal-Wallis Test Summary (Heartbeats, Days 1 through 4)				
Day	Total N	Chi-squared	Degrees of Freedom	Asymptotic Sig (2-sided test)
1	188	11.969	4	0.018*
3	185	24.011	4	0.000*
4	183	11.923	4	0.018*

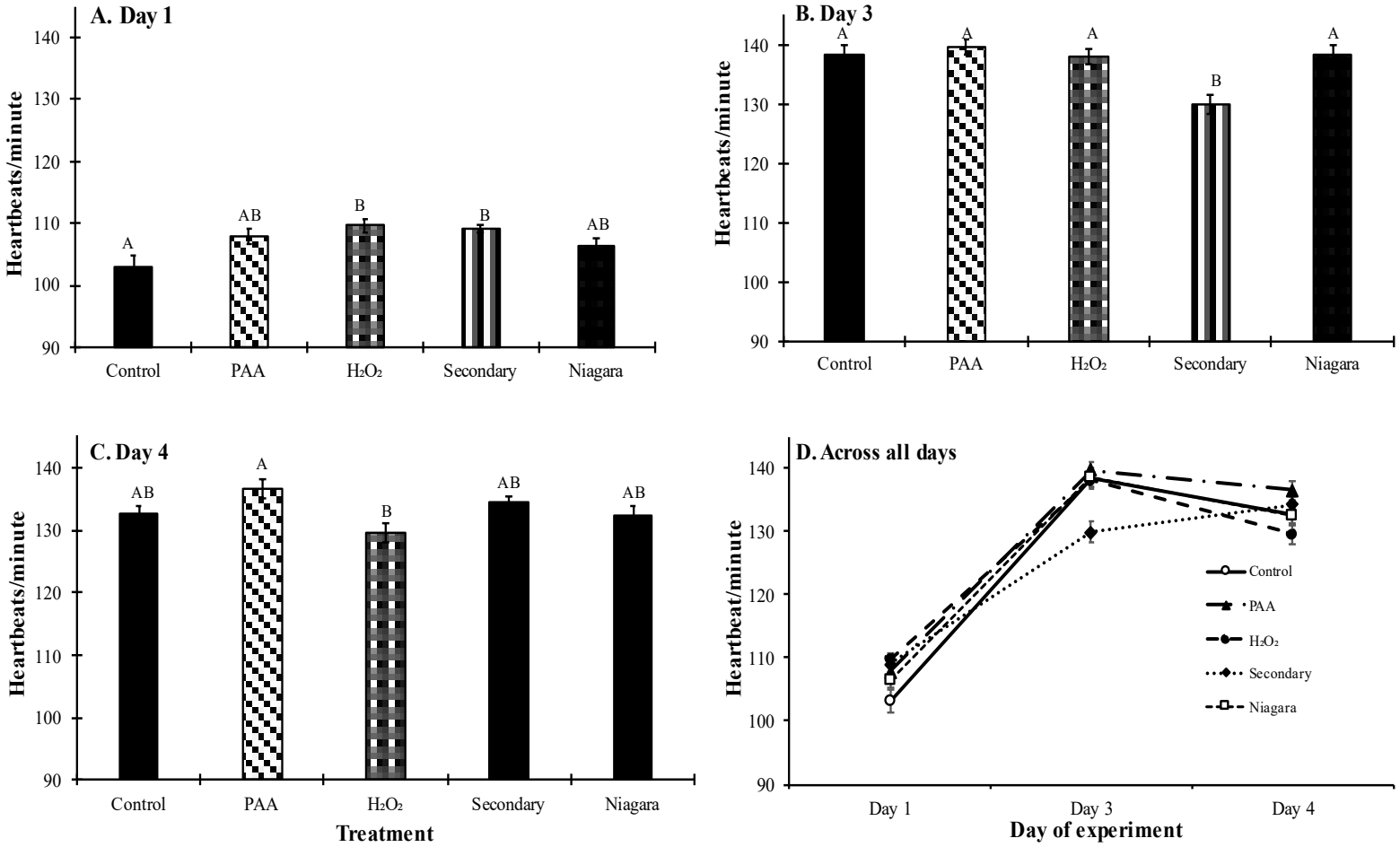

Figure 8. A-D. Heartbeats/minute of fathead minnow embryos by day (Mean ± SE). **(A)** Day 1 **(B)** Day 3 **(C)** day 4, and **(D)** embryo heartbeats across days. Letters above columns indicate significant differences between treatments (Dunn's test, Bonferroni correction, $p < 0.05$).

Hatching Success and Hatching Rate

The percentage of larvae that hatched from fertilized eggs was 100% for all treatments. the hatching rate was determined by the number of eggs hatched on day five compared to the total number of eggs. All treatments were compared to the Control (*hatching rate* = 81.2%) using a binomial test. Hatching rate was significantly greater in the PAA, H_2O_2, and Niagara

treatments compared to the Control (Binomial, $p < 0.01$, Table 3, Figure 9). The hatching rate was significantly lower in the Secondary effluent compared to the Control (Binomial, $p < 0.01$, Table 3, Figure 9).

Table 3: Binomial Distribution of hatching of fathead minnow eggs on Day 5. The probability was determined by comparing the Control to each treatment. Star indicates significance ($p < 0.05$).

Hatching Rate (at day 5)				
Treatment	Hatched	Unhatched	Hatching rate	P (from binomial significance test)
Control	30	7	0.811	-
PAA	36	1	0.973	0.004*
H_2O_2	35	1	0.972	0.005*
Secondary effluent	27	11	0.711	0.046*
Niagara	36	1	0.973	0.004*

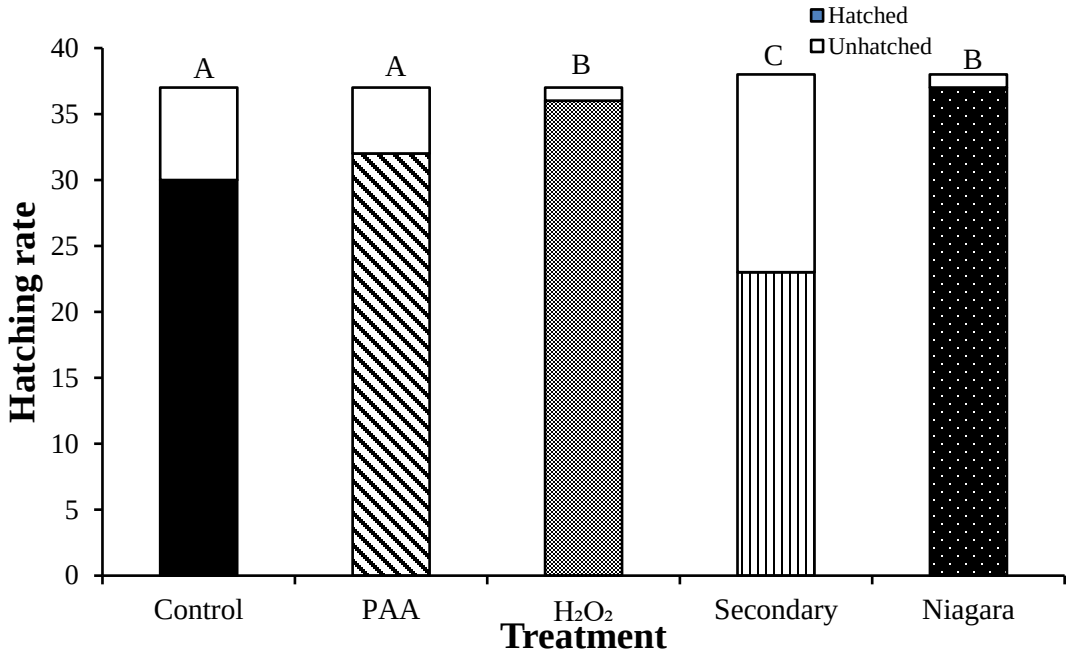

Figure 9. Hatching rate of fathead minnow eggs on day five. Different letters above the columns indicate statistical differences (Binomial, $p < 0.05$).

Abnormality Score

Freshly hatched fathead minnows were examined and given an abnormality score from 1 to 4 (1 = no abnormality, 2 = slightly abnormal, 3 = moderately abnormal, 4 = highly abnormal) (Figure 10). Fathead minnows in all treatments were predominantly normal (score of 1), and there was no significant difference in abnormalities between treatments (Kruskal-Wallis, $p = 0.489$). However, the average scores differed slightly among treatments. The Secondary effluent had a trend for the highest number of abnormalities (1.13 ± 0.06), followed by the Niagara treatment (1.11 ± 0.05). Fathead minnows in the Control (1.02 ± 0.03), H_2O_2 (1.02 ± 0.03), and PAA (1.05 ± 0.04) had the lowest abnormality scores.

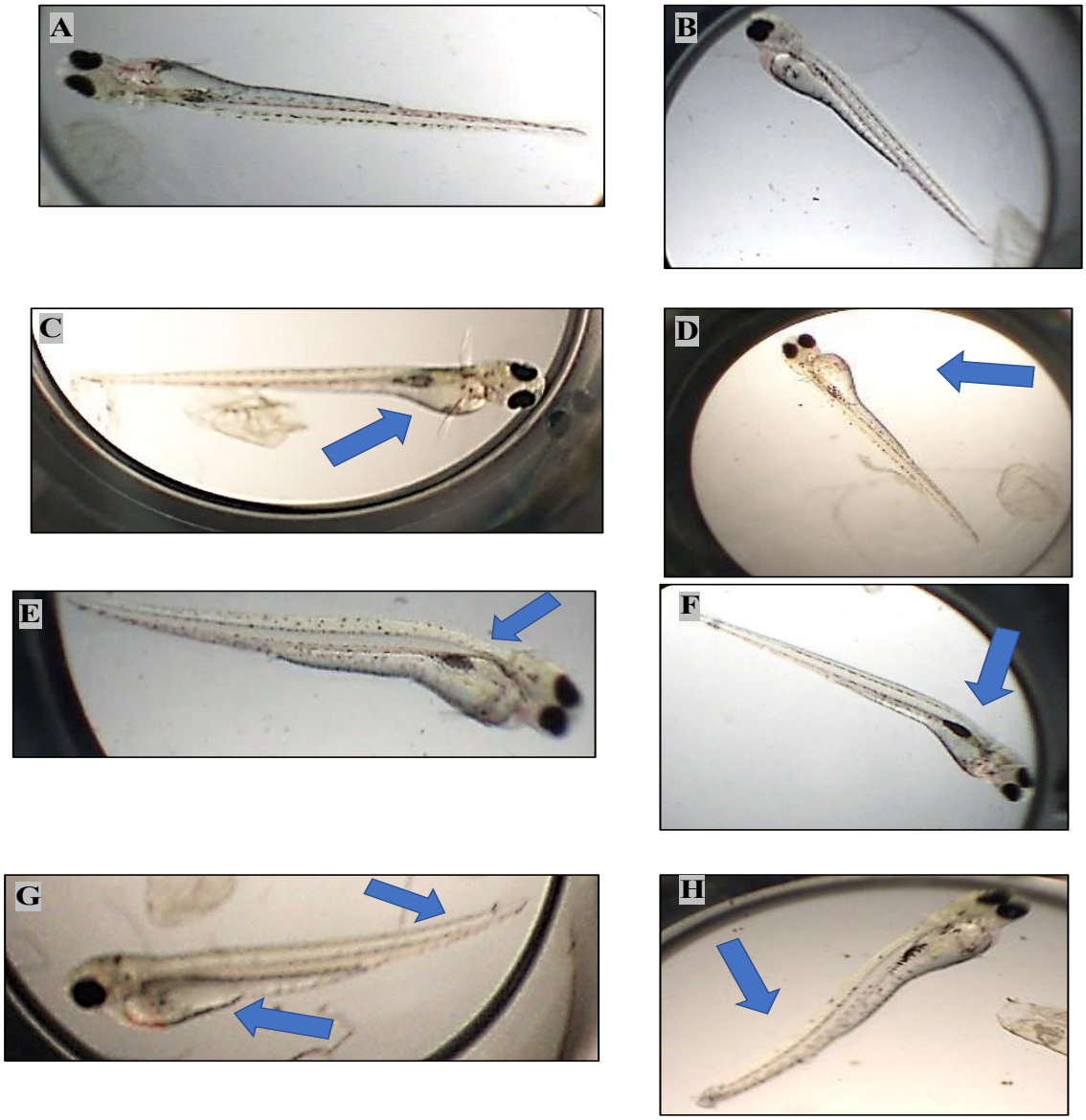

Figure10. Photos of fish taken during abnormality score processing. (**A,** Control) and (**B,** Niagara) are reference photos of normal fish that received an abnormality score of 1 (normal). Fish **(C-H)** each received an abnormality score of 2 (slightly abnormal). Fish (**C,** PAA) and (**D,** PAA) had slight edema. Fish (**E,** Niagara) had a slight curvature of the spine in the head region and slight edema. Fish **F** (Secondary effluent) has a curved of the spine in the head region. Fish (**G,** Control) has a slight upward curvature of the spine in the head region and slight edema. Fish (**H,** Secondary effluent) had slight curvature in the caudal region.

3.2 C-start Response

The endpoints determined by the C-start assay endpoints: *latency period, escape velocity, total escape, and escape angle*, were not statistically different between treatments ((ANOVA, significant $p < 0.05$, Table 4, Figure 11).

Table 4: Multivariate and Univariate ANOVA table for C-start endpoints. Larvae were 12 and 13 days old.

Endpoint	**Group**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig. (p)**
Latency Period	Between Groups	0.094	4	0.024	0.345	0.847
	Within Groups	4.132	61	0.068		
	Total	4.230	65			
Escape Angle	Between Groups	0.042	4	0.10	0.656	0.625
	Within Groups	0.972	61	0.016		
	Total	1.014	65			
Escape Velocity	Between Groups	0.600	4	0.150	2.059	0.097
	Within Groups	4.442	61	0.073		
	Total	5.041	65			
Total Escape	Between Groups	0.673	4	0.168	1.321	0.272
	Within Groups	7.768	61	0.127		
	Total	8.441	65			

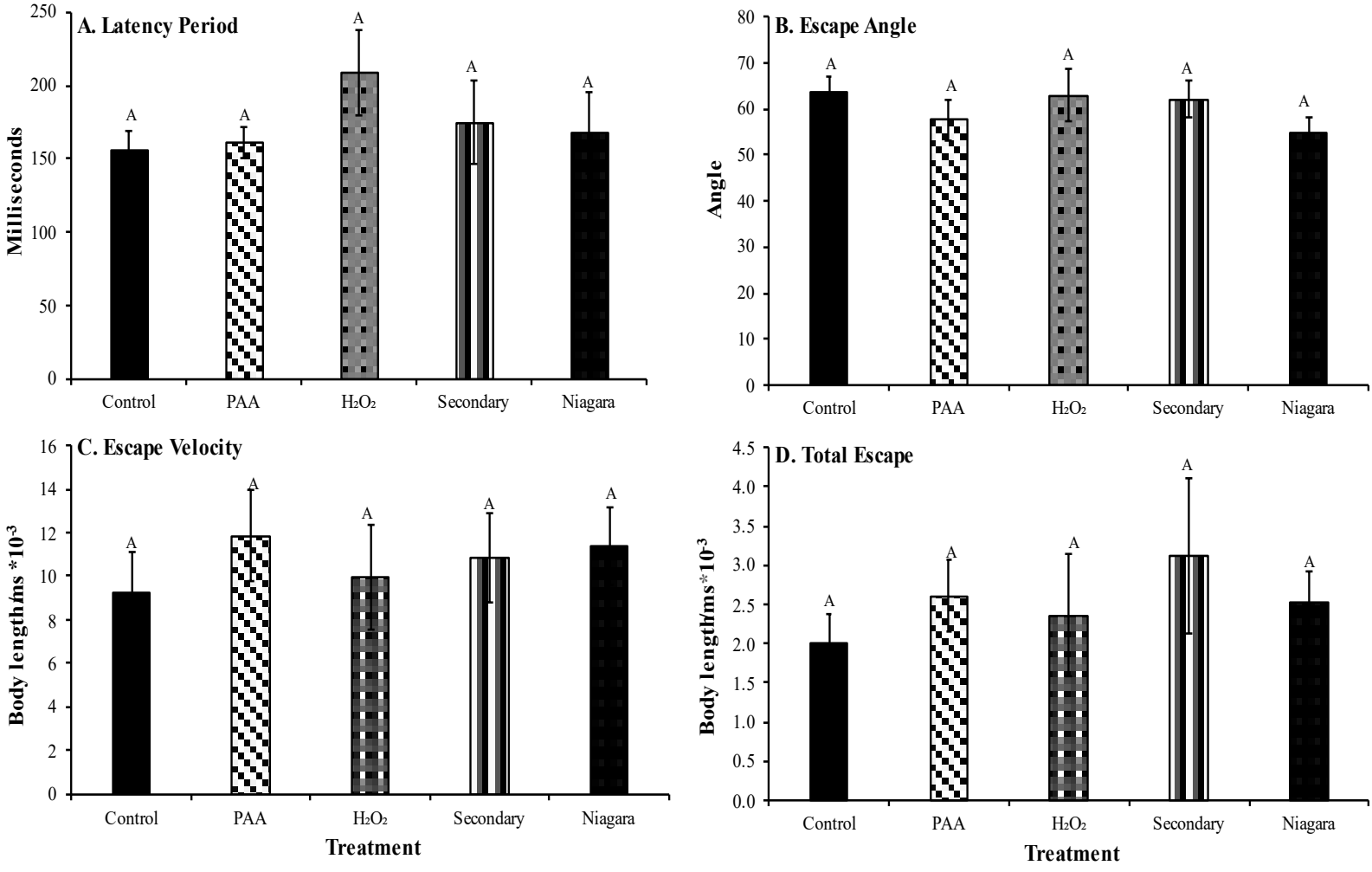

Figure 11. Effects of exposure to AOPs on predator avoidance performance in larval fathead minnows. **(A)** Mean of Latency Period, time milliseconds (ms) from initiation of stimulus to first head movement of C-start response. **(B)** Mean Escape Velocity for 42.857 ms after first head movement of C-start measured in body length per ms **(C)** Mean Escape Angle, angle of the deepest measured bend of the C-start response. **(D)** Mean Total Escape Response from stimulus until 42.857 ms after first head movement of C-start. Lack of different letters above the column indicates no significant differences (ANOVA, $p < 0.05$).

Table 5: Univariate ANOVAs for weight, length, and Fulton's Condition Factor of larval fathead minnows 12 and 13 days old.

Endpoint	Group	Sum of Squares	Df	Mean Square	F	Sig. (p)
Larval Length	Between Groups	1.694	4	0.423	0.889	0.475
	Within Groups	35.263	74	0.477		
	Total	36.957	78			
Larval Weights	Between Groups	7.365	4	1.841	2.554	0.046*
	Within Groups	54.075	75	0.721		
	Total	61.440	79			
Fulton's Condition Factor	Between Groups	0.029	4	0.007	1.694	0.160
	Within Groups	0.318	74	0.004		
	Total	0.347	78			

Larval weights at day 12 and 13 were significantly different. Larvae in the Secondary effluent were the heaviest, and significantly heavier than the Niagara (ANOVA, $F = 2.26$, $p < 0.05$, Table 5, Figure 12). The Control, H_2O_2, and PAA larvae were of similar weights.

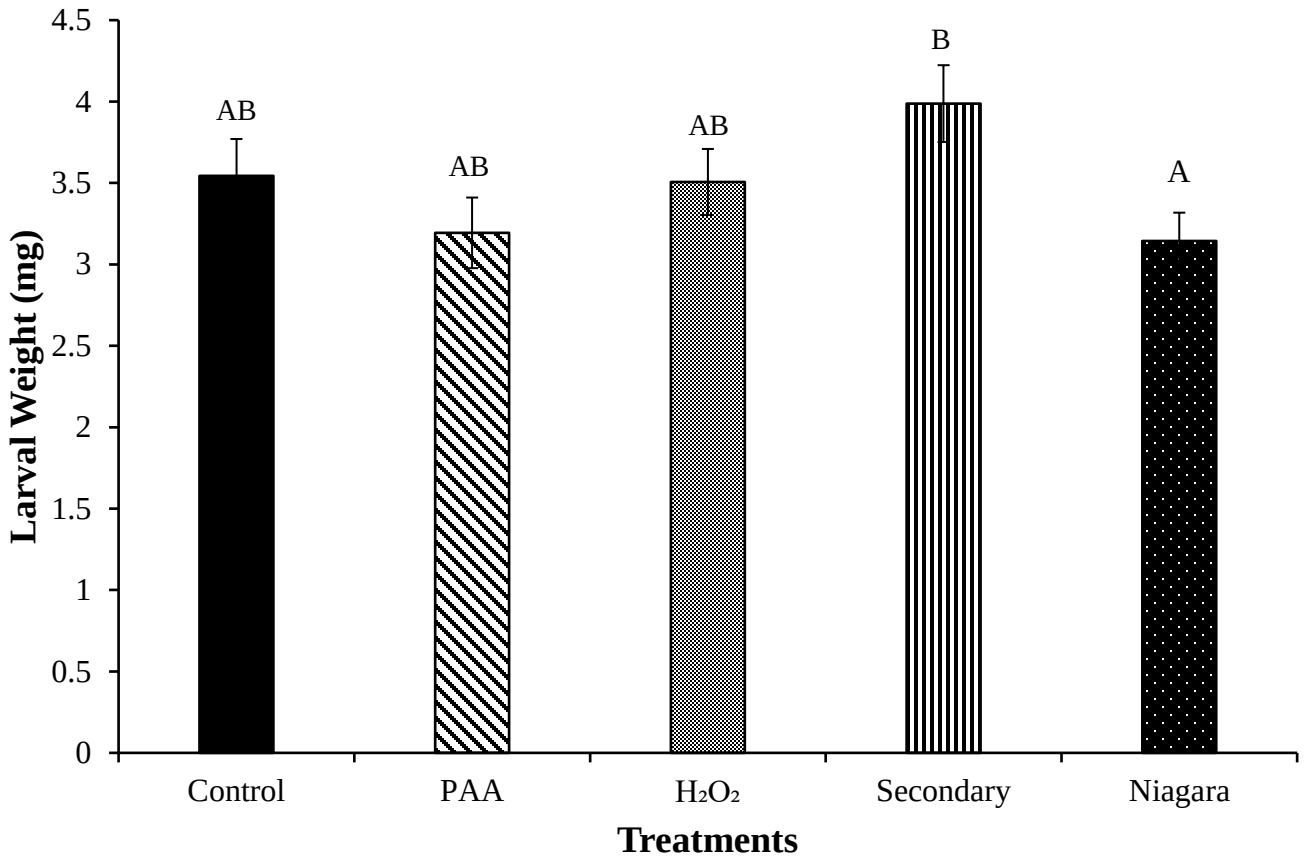

Figure 12. Mean ±SE wet weight of larval fathead minnows (mg) taken on days 12 and 13. Letters above the columns indicates significant differences (*post-hoc* Tukey HSD, $p < 0.05$).

Larval lengths were not statistically different on days 12 and 13 (ANOVA, $F = 1.11$, $p > 0.05$, Table 5, Figure 13), however the Secondary effluent larvae had the greatest length, followed by the Control, H_2O_2, Niagara, and the PAA larvae.

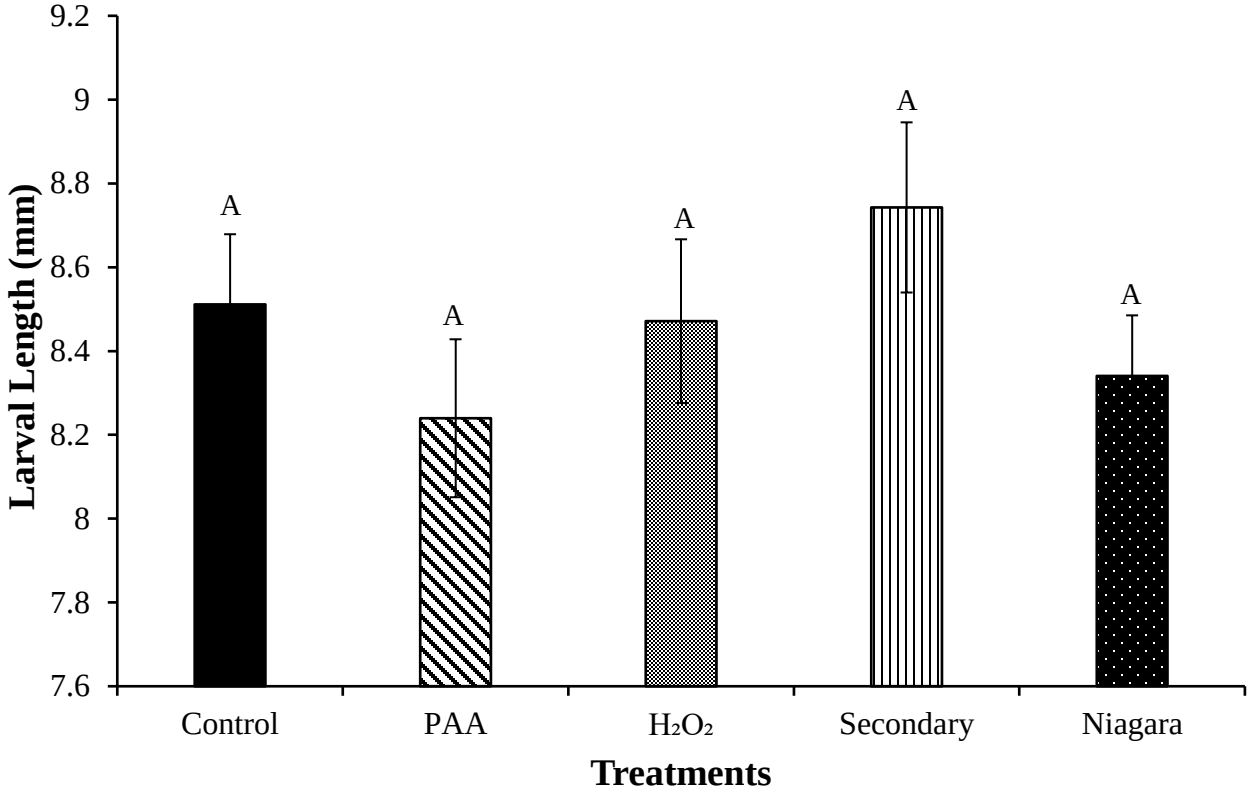

Figure 13. Mean ± SE length of larval fathead minnows (mm). All measurements were taken when larvae were 12 or 13 days old. Letters above the columns indicates significant differences (*post-hoc* Tukey HSD, $p < 0.05$).

Fulton's condition factor indicates if a fish's length is proportional to its length. There was no significant difference in Fulton's condition factor between treatments (ANOVA, $F = 1.636$, $p > 0.05$, Table 5, Figure 14). However, there was a trend for Secondary effluent's fathead minnows to have the highest condition factor, followed by the H_2O_2 and then the Control. The PAA and Niagara larvae had the lowest values.

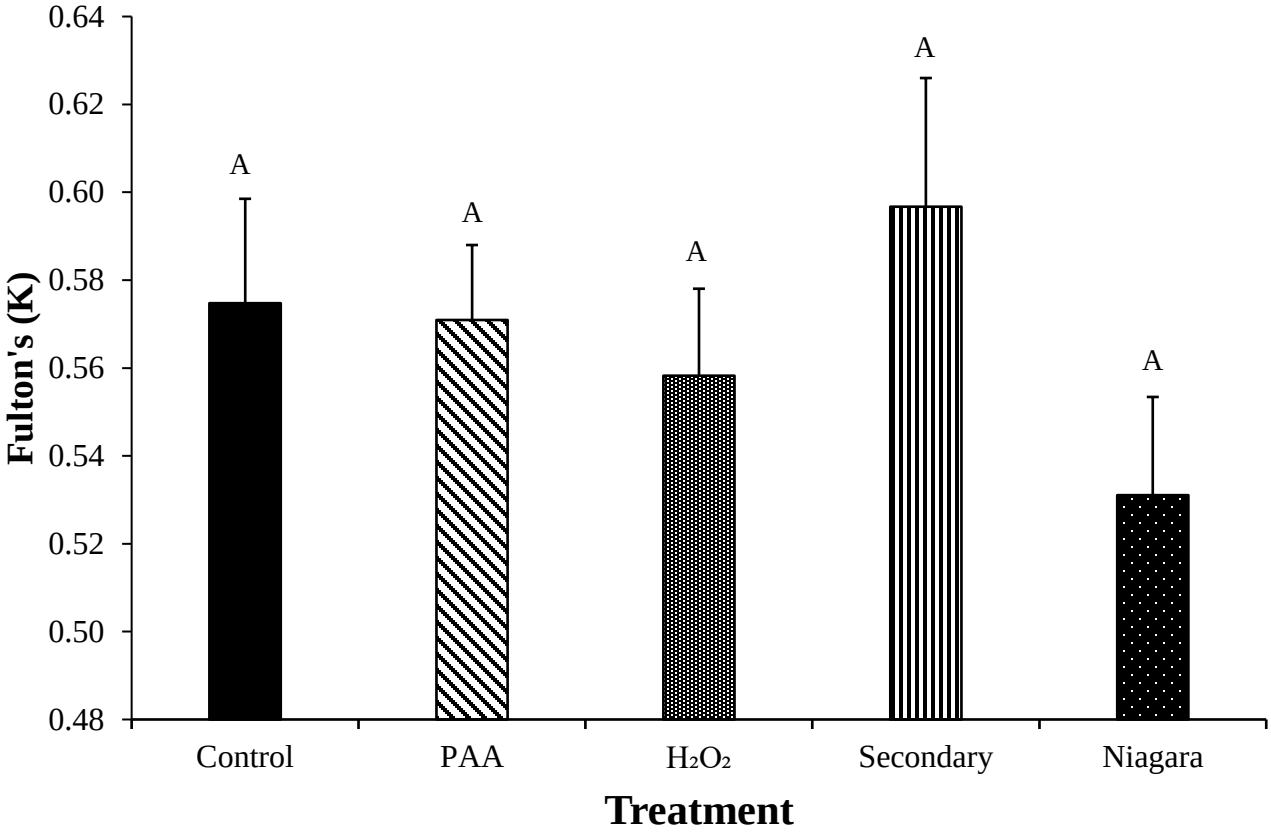

Figure 14. Fulton's Condition Factor with weight and length taken on day 12 and 13. Letters above the columns indicates significant differences (*post-hoc* Tukey HSD, $p < 0.05$).

Relative Growth Pattern (*b*) was determined for each treatment by using power curves of weight and length (Figure 15). The PAA treatment fish had the highest *b* (3.10), indicating they were the plumpest, and in turn healthy (Froese 2006). The next highest b value was for larvae in the Control (2.81), followed by the Niagara larvae (2.79). The H_2O_2 (2.32) and the Secondary effluent (2.018) larvae had the lowest b-values (Figure 16).

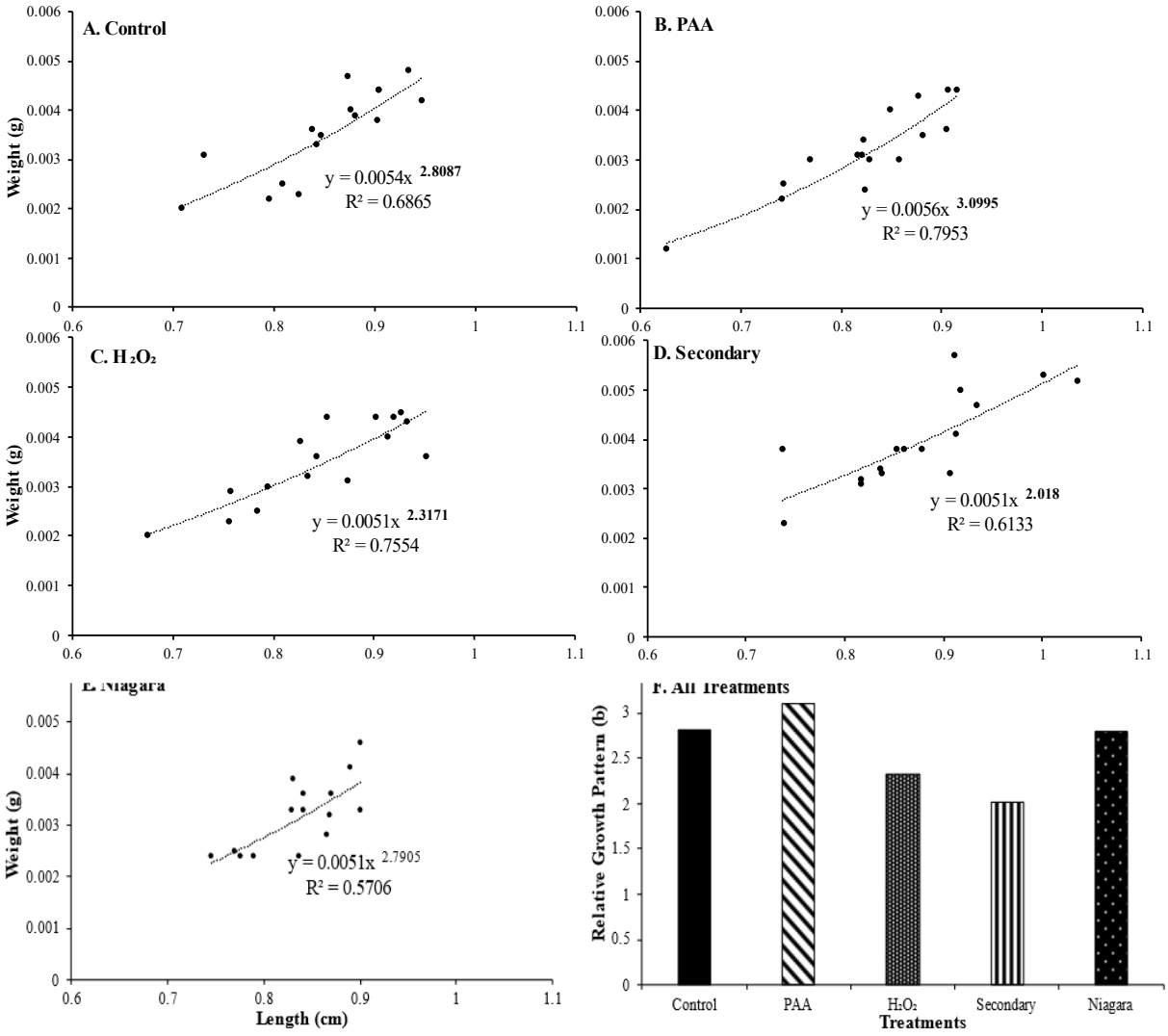

Figure 15A-F. Power curves were used to determine Relative Growth Pattern (b) for larval fish raised in different treatments. The bold exponent indicates *b* for each treatment. **F** is the relative growth pattern for each treatment.

C-start Pearson's Correlation and Principal Component Analysis

A Pearson's correlation matrix was used to compare C-start endpoints. When looking at all treatments combined, *escape velocity* and *escape angle* had a significant negative Pearson's correlation ($r = -0.560$, $p < 0.05$, Table 6). Additionally, *velocity* and *latency period* had a nearly significant negative correlation ($r = -0.188$, $p = 0.065$). *Velocity* and *latency period* had the most significant correlations between treatments. For example, *velocity* and *escape angle* were negatively correlated for larvae in the Control ($r = -0.-710$, $p <0.05$), H_2O_2 ($r = 0.455$, $p < 0.001$), and Niagara treatments ($r = -0.538$, $p <0.05$). Additionally, *velocity* and *latency period* showed positive correlation in the H_2O_2 treatment ($r = 0.455$, $p < 0.001$). *Latency period* and Fulton's condition factor was correlated for PAA larvae ($r = 0.493$, $p < 0.05$), while *latency period* and *escape angle* were correlated for Secondary effluent larvae ($r = - 0.501$, $p < 0.05$).

Table 6: Pearson Correlation Matrix for the condition factors and C-Start response of larval fish exposed to the different treatments. Pearson correlations are displayed above the grey boxes and p-values are below the grey boxes, asterisks indicate $p < 0.05$, - indicates not significant.

Treatment	Endpoint	Angle	Latency	Velocity	Fulton
All Treatments Combined	Angle		-0.038	-0.560	0.055
	latency	-		-0.188	-0.088
	velocity	*0.000	0.065		0.039
	Fulton	-	-	-	
Control	Angle		-0.289	-0.710	-0.333
	latency	-		-0.075	-0.349
	Velocity	*0.002	-		0.337
	Fulton	-	-	-	
PAA	Angle		-0.108	-0.422	0.241
	Latency	-		-0.180	0.493
	Velocity	0.066	-		0.094
	Fulton	-	0.037*	-	
H_2O_2	Angle		0.455	-0.832	0.261
	Latency	0.068		-0.741	-0.337
	Velocity	0.000*	0.003*		0.130
	Fulton	-	-	-	
Secondary effluent	Angle		-0.501	-0.263	-0.155
	Latency	0.034*		-0.304	0.037
	Velocity	-	-		-0.328
	Fulton	-	-	-	
Niagara	Angle		0.159	-0.538	0.001
	Latency	-		0.422	-0.134
	Velocity	0.035*	0.086		0.047
	Fulton	-	-	-	

Principal component analysis found two principal components. Component 1 had an Eigenvalue of 1.61 and explained 40.33% of variance (PCA, varimax rotation, Figure 17). Component 1 showed velocity decreasing as angle increased. Component 2 had an Eigenvalue of 1.21 and explained 30.18% of variance (PCA, varimax rotation, Figure 17). Component 2 showed latency period decreasing, as Fulton's condition factor increased. Together, principal components 1 and 2 comprised 70.5% of the variance. Figure 18 shows PCA, with further definition by treatment.

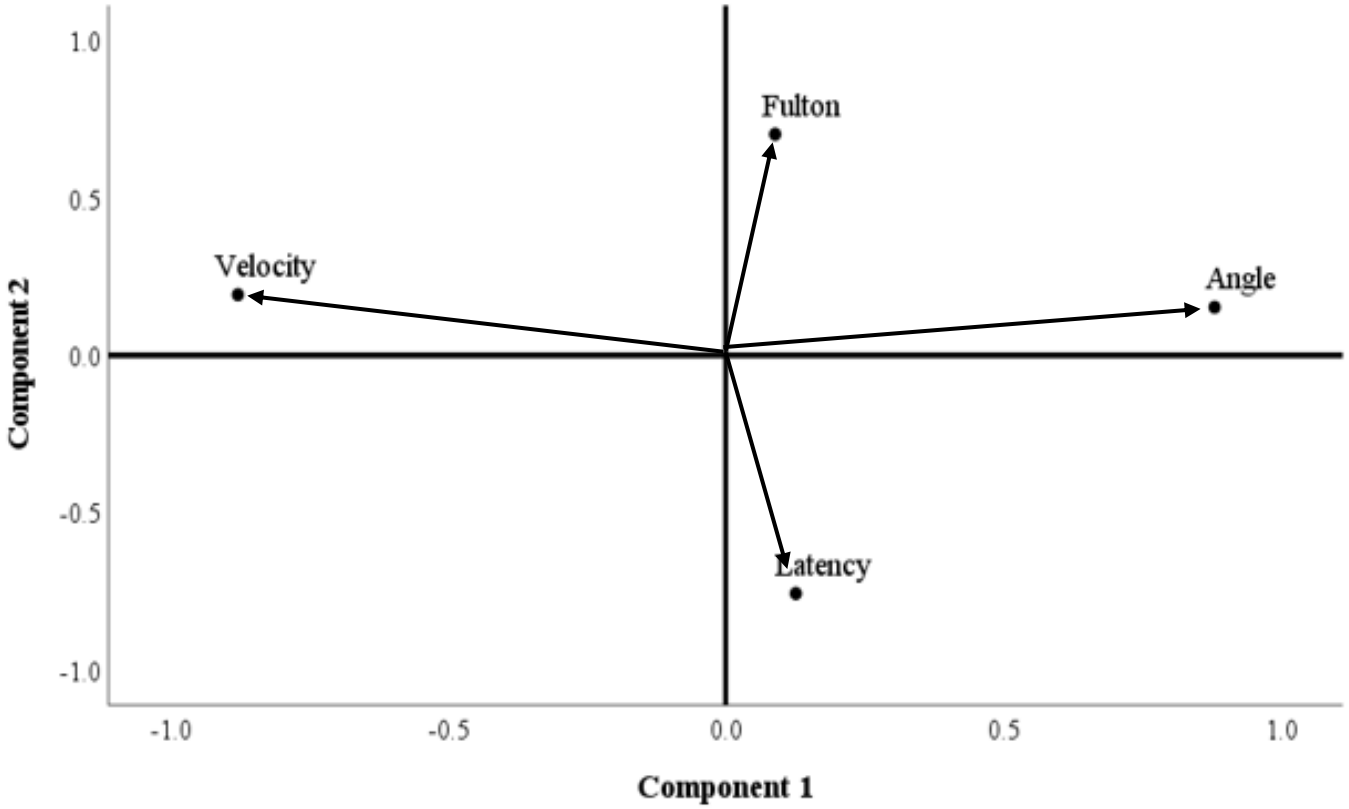

Figure 16. Principal component analysis with the Varimax rotation. All components are endpoints from the C-Start assay of 12 and 13-day old fathead minnows.

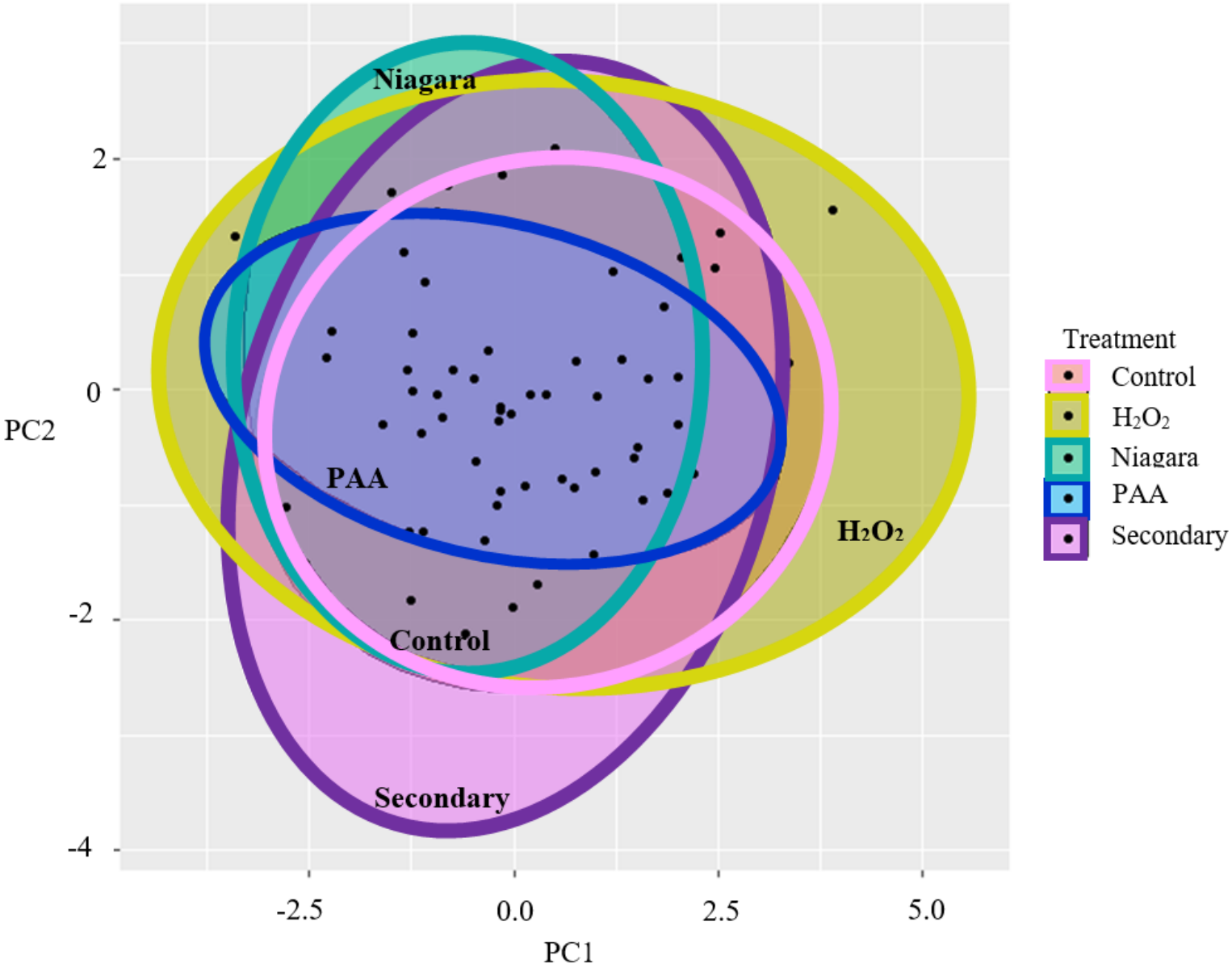

Figure 17. Principal component analysis of C-start endpoints. The ellipses highlight treatments as they pertain to components. All fathead minnows were 12 and 13-days old.

3.3 Juvenile Weight, Length, and Condition Factor

Wet weight and total length from age 30-days fathead minnow juveniles were used to calculate Fulton's condition factor (K) and Relative Growth Pattern (b) for the juvenile fish raised in the different treatments (Table 7).

Table 7: Univariate ANOVAs results for wet weight, total length, and Fulton's condition factor of 30-day old fathead minnows.

Endpoints	**Group**	**Sum of Squares**	**Df**	**Mean Square**	**F**	**Sig.**
Weight (mg)	Between Groups	561.7	4	140.42	6.04	0.0003*
	Within Groups	1580.9	68	23.25		
	Total	2142.6	72			
Length (mm)	Between Groups	27.38	4	6.845	5.77	0.0005*
	Within Groups	80.66	68	1.186		
	Total	108.04	72			
Fulton's Condition Factor (K)	Between Groups	0.0063	4	0.0016	1.440	0.230
	Within Groups	0.0753	69	0.0011		
	Total	0.0818	73	0.0027		

The final wet weights of juvenile fathead minnows were significantly different between treatments (ANOVA, $p < 0.001$, Table 7, Figure 18). Juvenile fathead minnows raised in the Control were significantly heavier than juveniles raised in PAA and H_2O_2 (*post-hoc*, Tukey HSD, $p < 0.05$, Figure 19). Juvenile weights in the Secondary effluent and Niagara treatments were lower than in the Control, but heavier than the AOP-treated effluents (Figure 18).

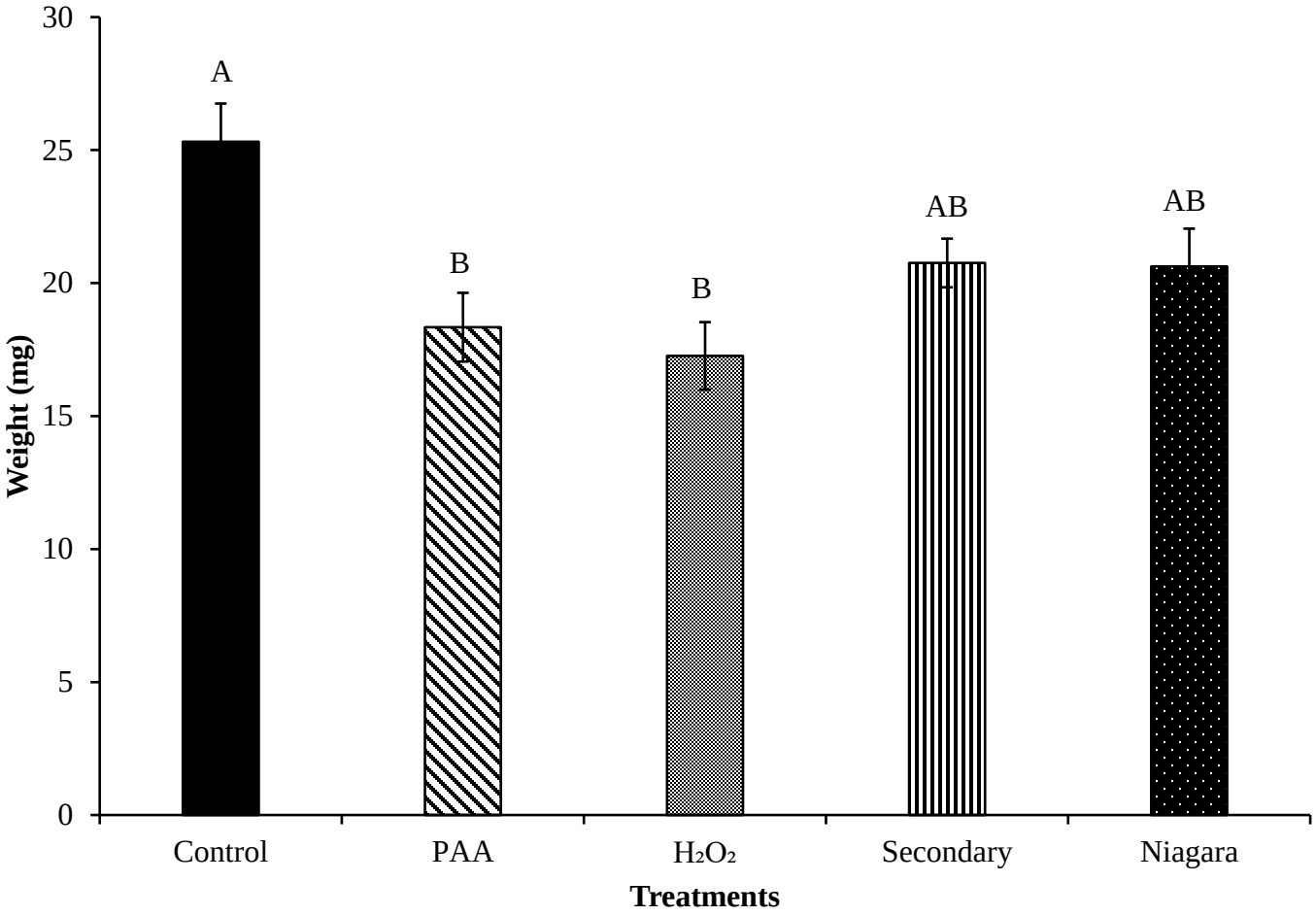

Figure 18. Wet weight of juvenile fathead minnows (mg). All measurements were taken on day 30. Different letters above the columns indicates significant differences (*post-hoc* Tukey HSD, p-value < 0.05).

There were differences in total length. The PAA and H_2O_2 fish had significantly smaller lengths than the Control juveniles (ANOVA, $p < 0.001$, *post hoc* Tukey HSD, $p < 0.05$, Table 7, Figure 19). The Secondary effluent and Niagara juveniles were most similar to one another and had lengths larger than the AOP-treated effluents.

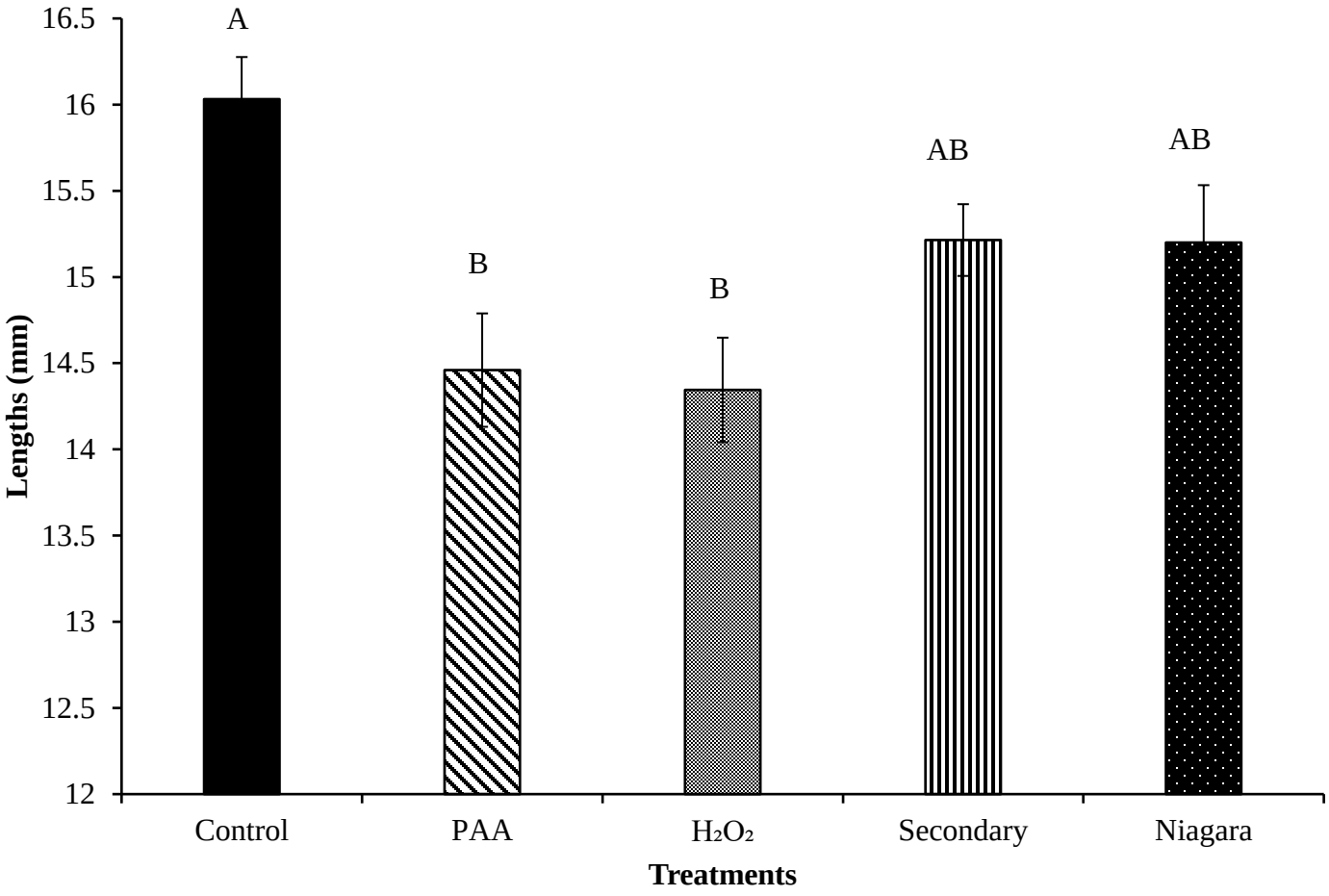

Figure 19. Mean total length of juvenile fathead minnows (mm) ± SE. All measurements were taken when fish were 30 days old. Different letters above the columns indicates significant differences (*post-hoc* Tukey HSD, p-value < 0.05).

At day 30, Fulton's condition factor did not differ statistically in any of the treatments (ANOVA, $p > 0.05$ Table 8, Figure 21), however, the H_2O_2 treatment had the lowest Fulton-value. The Control had the highest value, followed by the PAA, Secondary effluent, and Niagara treatments.

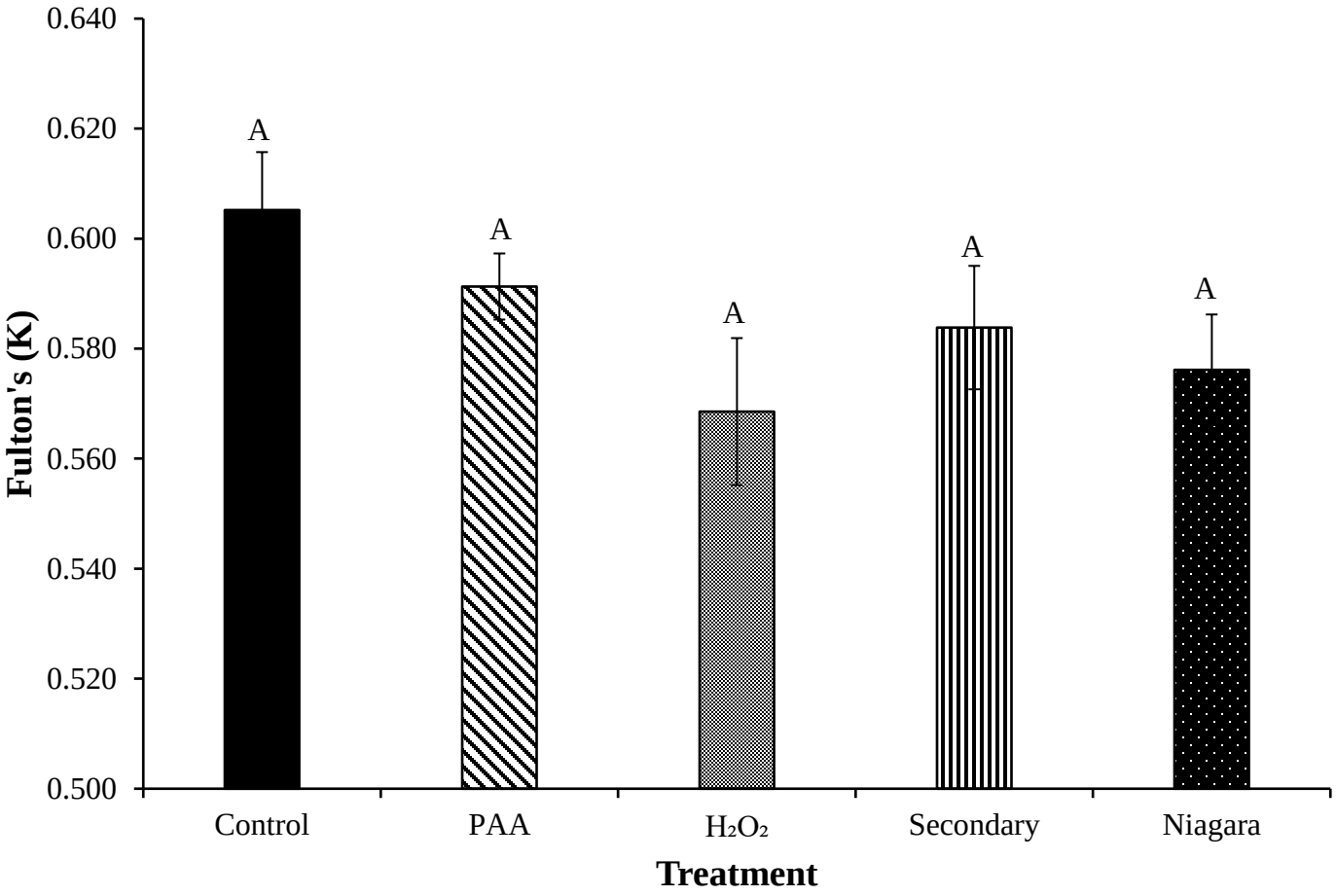

Figure 20. Fulton's condition factor (K) of 30-day old by treatment. Similar letters above columns indicates lack of statistical difference between treatments (*post hoc* Tukey HSD, $p < 0.05$).

The Relative Growth pattern (b) at 30-days (Figure 21) showed that the highest b-value was in the Control (3.70) juveniles, followed by the H_2O_2 (3.44), the Niagara (3.27), the PAA (3.17), and lastly the Secondary effluent (2.87) juveniles. Juveniles in the Secondary effluent had the lowest value and was the only treatment with fish displaying allometric growth (b < 3) (Figures 21).

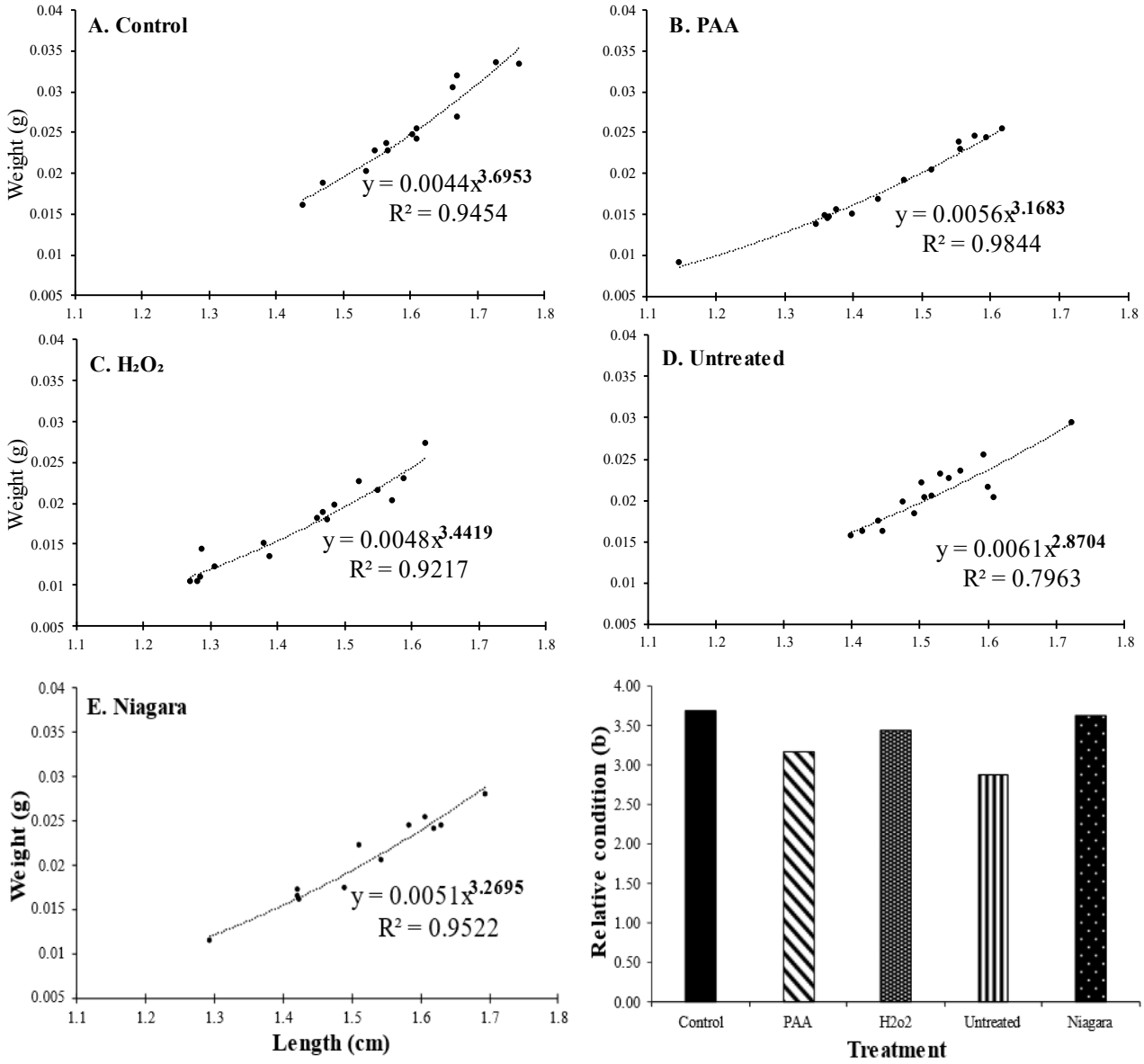

Figure 21 A-E. Relative Growth Pattern (*b*) was determined for 30-day-old fish using weight-length relationships. The bold exponent indicates *b* for each treatment. **F** is all *b-value* for all treatments.

Discussion

Exposing fish to potential toxins across life stages can highlight unique vulnerabilities in fish development. The experiments in this study were designed to take advantage of different life stages to determine the biological effects of AOP treated effluent on fish development. In the experiments, heartbeat and hatching time during the embryo stage indicated differences in how the embryos responded to treatments, while measurements of weight and length showed greater differences in the fish with prolonged exposure to the treatments.

Heartbeat as a Stress Indicator

When examining the response of embryo heartbeat to the different treatments, results reflected an altered heartbeat pattern compared to the Control, which indicates that the substances dissolved in these effluents caused stress to the embryo. The PAA, Niagara, and Control embryos had similar heart rates across the first four days of treatment. However, on day one, the Secondary effluent and H_2O_2 embryos had significantly higher heart rates than the Control (Table 2, Figure 8). This initial spike likely was a stress response that results from impaired aerobic metabolism (Gouva et al, 2020). In general, embryo heart rate increases as a function of development, and a decreased heart rate signifies retardation in development (Li et al. 2008). On day three, the Secondary effluent embryos had a significantly lower heart rate than the embryos in all the other treatments, while H_2O_2 embryos had the lowest heart rate on day four. One potential cause for these lower heart rates could be the presence of anticonvulsants, specifically carbamazepine, diclofenac, and lamotrigine. These were present in all effluent treatments (Figure 1) however, the concentrations were much higher in the secondary effluent, providing a clue that the AOP treatments did have a positive effect in fish embryo heartbeats.

Anticonvulsant medications are designed to block ion channels (Azarbayjani and Danielsson 1998), which are critical for all aspects of cardiac function including rhythmicity and contractility. Thus, it is possible that off-target interactions of PPCPs with cardiac ion channels may have caused the decrease in heartbeat rates observed in the Secondary and H_2O_2 treatments (Priest and McDermott 2015).

Worthy of note is that the Secondary effluent and H_2O_2 embryos had a decreased heart rate while the PAA treatment did not (Table 2, Figure 8). This is despite the presence of some level of anticonvulsants in all three effluent treatments. A possible explanation for this may be in the egg chorion, which is the outermost membrane that surrounds the embryo and is particularly sensitive to hydrogen peroxide (Rach et al. 1997, Stephenson et al. 2011, Fend et al. 2019). Hydrogen peroxide can accelerate chorion thinning (Rach et al. 1997) and break down fibrous lamellae in the internus layer (Figure 213) (Stephenson et al. 2011). An impaired chorion is likely less protective and exposed embryos in the H_2O_2 treatment to a greater concentration of PPCPs compared to the PAA treatment (Li et al. 2018, Kolmijeca et al. 2020).

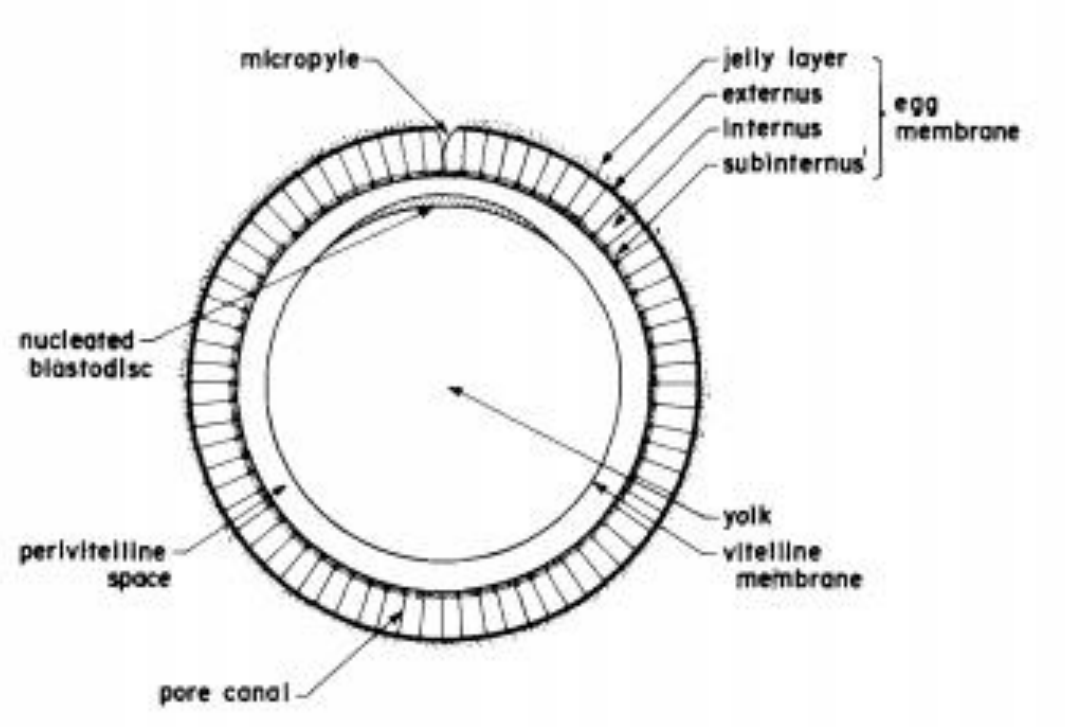

Figure 22. Schematic diagram of a fertilized and water-activated salmonid egg. Note the arrow pointing to the upper right egg membrane to highlight the jelly layer, externus, internus, and subinternus (Groot and Alderdice, 1985).

Hatching Success and Hatching Rate

All treatments had 100% hatching success, yet the hatching rate for each treatment was significantly different than the Control. Ninety-seven percent of eggs raised in the PAA, H_2O_2, and Niagara treatments hatched on day five, compared to 81% in the Control, and 71% in the Untreated effluent (Table 3, Figure 9). Differences in hatching rate equate to a shorter hatching time in the PAA, H_2O_2, and Niagara treatments, and a longer hatching time in the Secondary effluent. A shorter hatching time is ecologically meaningful because many teleost species prey on the eggs of their own species. For instance, a male fathead minnow might consume some or all of the eggs that he is guarding, with egg consumption increasing as male condition decreases

due to energy being put into defending the nest instead of foraging (Konodoh and Okuda 2002). Furthermore, female and non-breeding male fathead minnows are voracious egg eaters (Green et al. 2008), hence, the longer the eggs remain adhered to the nest, the greater their chances of being consumed. Interestingly, the eggs exposed to the PAA, H_2O_2, and Niagara treatments hatched more quickly than the eggs in the Control. Ecologically, the biggest risk of early hatching is for the larvae to encounter lower water temperatures in the Spring, making it more difficult to swim due to lower muscle efficiency and increased viscous drag. Thus, fish larvae that have been exposed to AOPs might experience early hatching rates and encounter unfavorable environmental conditions (Von Herbring 2002). Though in fathead minnow early hatching equated to just one day, in fish with longer gestation periods, such as walleye (*Sander vitreus)* with a gestation period of 26 days (fishbase.org), early hatching could have a more significant effect.

An interesting result related to hatching time occurred in the H_2O_2 treatment. Although these embryos had a decreased heart rate on day four, there was a shorter hatching time than the Control. Hydrogen peroxide weakens the chorion, which likely made it easier for fish to break out of the egg (Stephenson et al. 2012, Figure 24). Embryos induce hatching by dissolving the inner layer of the chorion with hatching enzymes secreted from the epithelium of the mouth, pharyngeal cavities, and the surface of the operculum (Suga 1963). Considering hydrogen peroxide is known to breakdown lamellae within the inner layer (Stephenson et al. 2012), the chorion may have been easier to break out of, and this would explain how fish with the lowest heart rate on the day prior to hatching still had a significantly shorter hatching time than the Control.

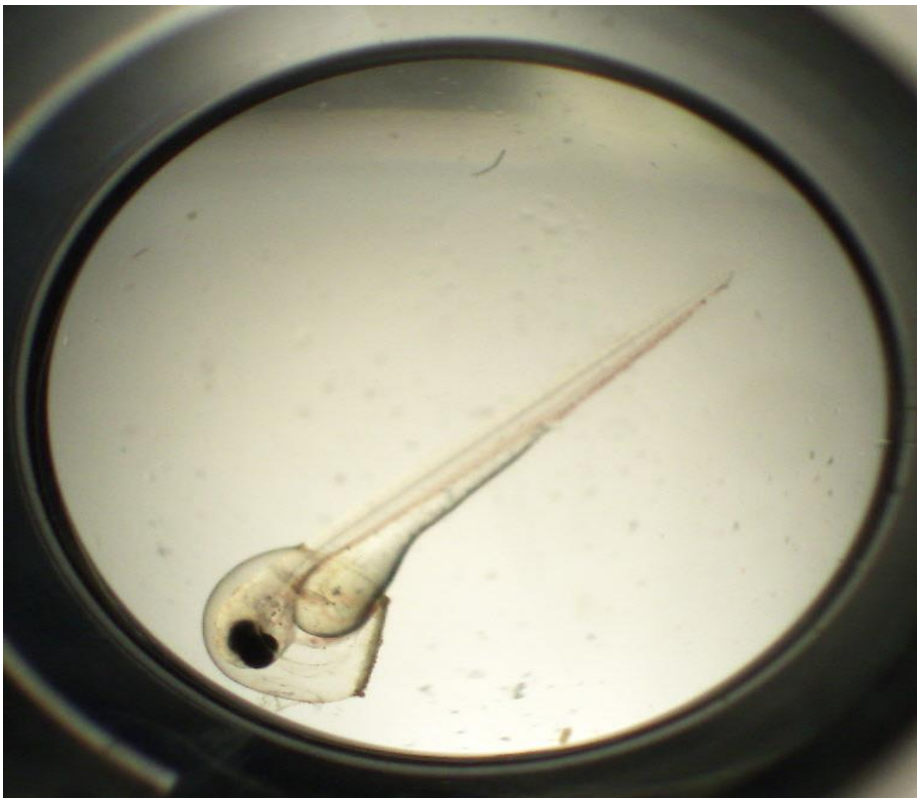

Figure 23. Hatched fathead minnow in the H_2O_2 treatment with the shell still surrounding the head region. Shell was around the head for about two hours (*personal observation*).

Body Morphology and Treatment-Induced Abnormalities

Body morphology was not significantly different between treatments. However, Secondary effluent and Niagara larvae tended to have more abnormalities than the Control, PAA, and H_2O_2 larvae. It is not surprising that the Secondary effluent had more abnormalities considering the greater concentration of PPCPs (Figure 1). The Niagara also had a large number of PPCPs (Arnnok et al. 2017), which may explain the higher trend for morphological abnormalities. While hydrogen peroxide does negatively impact chorion structure, the H_2O_2 treatment is effective at removing PPCPs (Figure 1). This lower amount of PPCPs could explain the lower number of abnormalities in the larvae in the H_2O_2 treatment. For the PAA treatment,

the lack of abnormalities in the larvae paired with a heart rate more similar to the Control indicates that the PAA treatment was less harmful to fishes' early life stages than H_2O_2 or Secondary effluent.

C-Start Response

In regard to the C-start response in young larvae (12 to 13 days old), multivariate analysis found no significant comparisons between *latency period, escape angle, velocity,* and *total escape*. When these endpoints were analyzed individually using ANOVA, there were also no significant differences. These results were unexpected given the presence of the PPCPs sertraline, bupropion and venlafaxine in all effluent treatments, which are known to decrease C-start performance (Painter et al. 2009, Thompson and Viajan 2020).

The C-Start is controlled by special neurons known as Mauthner cells. Mauthner cell axons extend into the spinal cord, stimulate primary and secondary neurons, and then excite the lateral musculature (Painter, 2009). The lack of significant differences in the C-start response may indicate that there is not a large enough toxicant burden in any of the effluent treatments to interfere with these neurotransmitters or their receptors (Painter et al. 2009). It is important to keep in mind that fish were only 12 and 13 days old at the time of this study. As fish age, PPCPs bioaccumulate within tissues, including brain tissue (Arnnok et al. 2017). A predator avoidance test carried out after a longer period of exposure may be a better indicator of predator avoidance behavior of fish who live in effluent-receiving bodies of water. Perhaps a prolonged swim test could provide more insight into the spectrum of effects that effluents could have on larval swimming behavior. The C-start is a reflex enacted by faster (white) muscle fibers and anaerobic respiration (Jayne and Lauder 1994; Weber and Haman 1996). In contrast, prolonged swim tests

use slow twitch ‘red’ muscle, which uses aerobic respiration (McPhee and Janz 2014; Teulier et al. 2019). Swim tests that utilize aerobic respiration are more sensitive to low-level toxicants. For instance, in a study that examined the impact of heavy metals in yellow perch (*Perca flavescens),* critical swimming speed was impaired while fast start swimming performance showed no difference (Rajotte and Coutoure, 2002).

Length, Weight, and Condition

Although there was no significant difference in larval length between treatments at age 12 and 13 days, weights were significantly different. Larvae raised in Secondary effluent had the highest weight and Niagara larvae had the lowest weight. A potential explanation for the increased weight in Secondary effluent larvae may have been due to hormesis, an enhancement or stimulation of a physiological process by exposure to concentrations of toxins (Betinger and McCaughley 2002). Hormesis has also been observed in adult fathead minnows exposed to bleached sulfite mill effluent. Yet those same fish had severely altered secondary sexual characteristics (e.g., males growing ovipositors and females displaying nuptial tubercules and dorsal fin dots), indicating that increased growth does not always imply greater health (Parrot et al. 2002).

Fulton's condition factor was not significantly different among the fish in the various treatments. However, the fish in the Secondary effluent tended to have the highest condition factor. Fulton’s condition factor assumes that the weight of a fish is proportional to the cube of its length (Eq. 1). The fish in the PAA treatment were the only ones with a growth proportion/growth pattern (*b*) of 3, with the Control having the second-highest growth pattern (*b* = 2.8) (Figure 11). The Secondary effluent larvae had the smallest *b* (2.0) and were the

"thinnest". Growth pattern (*b*) indicates that though the Secondary effluent larvae were the longest and the heaviest, they were not necessarily the healthiest. Daily exposure to contaminants is energetically demanding and as fish remain stressed without a break from exposure, cortisol levels rise and enzymatic activity increases in order to maintain basal metabolism (Ings et al. 2012). Conversely, PAA larvae had the highest b-value which indicates that the PAA treatment is likely the safest effluent treatment for larval life stages.

Juvenile fish 30 days-old in the AOP treatments, PAA and H_2O_2, had significantly lower weights and lengths than the juvenile fish in the Control. These two treatments also had lower weights than the juveniles in the Secondary effluent and Niagara treatments (Table 7, Figures 19 &20). Both peracetic acid and hydrogen peroxide are oxidizing agents and have been shown to cause lesions in the gills, which are characterized by lamellar fusion, pillar cell necrosis, and pillar channel aneurysm (Stephenson et al. 2012). Speare et al. (1999) reasoned that as fish become larger, their gill surface area increases and they have more exposure to toxins, becoming more vulnerable to their effects. Future experiments should extend into the adult life stage and gill tissue should be inspected for damaged lamellae. Though the AOP-treatments were quenched to remove oxidants, it is possible that some oxidizing agents remained in the treatment and caused lesions to gill tissue, which in turn stunted growth. Decreased larval length is strongly correlated with decreased survival in aquatic environments (Zabel and Achord, 1999) and can have severe effects on the local food web.

Similar to the larval stage, when examining growth patterns (b) in the juvenile fish, the Secondary effluent fish were the skinniest. Surprisingly, though the H_2O_2 and PAA juveniles had the lowest weights and lengths, they had higher b-values than the Secondary effluent and Niagara treatments (Figures 22 & 23). This finding may indicate that growth is being stunted

while swimming and foraging behavior is not negatively affected (Bird 2015). At the juvenile or adult stage, future researchers should use a feeding efficiency test to determine the effects treatments have on a fish's ability to capture prey.

Conclusions and Future Research Recommendations

This set of experiments showed the value of testing potential solutions to the PPCP problem across life stages. During the embryo stage, the PAA treatment was more similar to the Control and Niagara, while embryos raised in the H_2O_2 treatment were more similar to the Secondary effluent. Heartbeats in the PAA and Control increased over time, while the H_2O_2 and Secondary effluent treatments showed an initial stress response on day one and slowed heartbeat on days 3 and 4 respectively (Figure 8). Slower heart rate was likely linked to delayed hatching time in the Secondary effluent, which puts eggs at a greater risk for predation, and further shows the need for better WWTP processes. Overall, the AOP-treated effluents decreased egg hatching time, which could put these larvae at a greater risk to the elements or predation.

Understanding the biological impact of AOP-treated effluent became more complicated as the fish matured. At the juvenile stage, fish in the H_2O_2 and PAA treatments had lower weights (Figure 18) and shorter lengths (Figure 19) than the fish in the Secondary effluent and Niagara treatments. Considering hydrogen peroxide and peracetic acid can harm gill tissue, the AOP procedure must be improved in order to remove more of the oxidants used. In examining juvenile condition factor (Figure 20), the PAA treatment aligned more closely with the Control while fish raised in the H_2O_2 were more similar to the Secondary and Niagara treatments. Taking into account the impact on heartbeats, hatching, and condition factor, the PAA appears to be the safer AOP for treating WWTP effluents.

Future studies should examine the impact of AOP-treated effluent on the adult life stage. As mentioned above, AOP-treated effluent should be analyzed for oxidants to better understand residual amounts. This is especially important considering the negative impact hydrogen peroxide has on chorion lamellae (Stephenson et al. 2012), and peracetic acid and hydrogen peroxide have on gill tissue. Thus, future studies should examine both the chorion structure during the embryo stage and gill tissue for lesions as a potential mechanism for growth impairment. Additionally, feeding efficiency assays should be implemented to better understand foraging behaviors and contextualize condition. For instance, if a fish has a low growth pattern but has no impairments with prey capture or appetite, it helps support the hypothesis that the energy is being used for detoxification.

Attention should also be paid to the relationship between the Niagara and Secondary effluents. The Niagara River receives effluent from a multitude of WWTPs. As the experiment progressed, the Niagara treatment fish's weight, length, condition factor, and *b*-values were more similar to the Secondary effluent. It is possible that the consequences of bioaccumulation were beginning to impact fish health in our study. Decreasing fish health as a function of extended time in the Niagara treatment further emphasizes the need to improve WWTP effluent procedures and decrease the PPCP load and other pollutants in aquatic communities.

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Appendix A

Table 1. Full name of PPCPs listed in Figure 1 as well as Concentration in ng/L (parts per trillion) in the different treatments.

	Name	Function	3 mg/L PAA-UV	6 mg/L PAA-UV	6 mg/L H2O2-UV	Field Station (Control)	Secondary Effluent
VEN	Venlafaxine	Antidepressant	119.40	104.86	105.22	0	164.31
PMD	Primidone	Anticonvulsant	52.20	50.08	51.78	0	70.30
LMT	Lamotrigine	Anticonvulsant	234.19	284.12	260.41	0	288.04
AZI	Azithromycin	Antibiotic	131.21	57.43	78.52	0	129.21
TMP	Trimethoprim	Antibiotic	169.19	149.85	150.25	0	209.34
CIP	Ciprofloxacin	Antibiotic	10.30	9.07	12.66	0	56.36
DES	Desvenlafaxine	Antidepressant	757.36	696.45	632.99	0	944.33
CBZ	Carbamazepine	Anticonvulsant	60.76	50.96	48.60	0	76.26
DIC	Diclofenac	Anti-inflammatory	0.00	0.00	0.00	0	5.72
CLA	Clarithromycin	Antibiotic	7.21	2.69	4.52	0	7.56
AMI	Amitriptyline	Antidepressant	15.46	8.82	10.18	0	22.53
SER	Sertraline	Antidepressant	23.74	10.96	13.07	0	27.63
CAF	Caffeine	Stimulant	35.83	24.41	30.80	40.81	22.09
IOP	Iopamidol	Contrast agent	273.09	345.93	320.86	0	3264.02
BUP	Bupropion	Antidepressant	111.04	76.44	83.86	0	160.63
CIT	Citalopram	Antidepressant	117.03	101.56	99.93	0	167.43
SMX	Sulfamethoxazole	Antibiotic	106.90	109.56	193.83	0	836.42
A-ERY	Anhydro-Erythromycin	Reagent	0.00	0.00	4.63	0	6.18