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Effects of 17α-ethinylestradiol and Density on Juvenile Fathead Minnow Survival and Body Size

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ABSTRACT

Anthropogenic changes have led to the increased use of wastewater treatment plants in stream systems near urbanized areas. Synthetic oral contraceptives, observed in wastewater treatment effluents, can cause negative effects on fish life history metrics. Previous exposures of 17α-ethinylestradiol (EE2) have been shown to affect survival and reproduction of fathead minnows (*Pimephales promelas*). However, density effects were not considered, and additional research is needed to examine the role of density among fish exposed to EE2. Multiple hypotheses indicate the interaction of density with contaminant exposure may ameliorate or exacerbate mortality. We examined how nominal EE2 concentrations of 0 ng/L, 5 ng/L, and 10 ng/L affect body size and mortality at various densities. Fish body size was influenced by density but not EE2 exposure. When density was high, we did not detect an effect of EE2 exposure on mortality. However, when density was low, EE2 exposures increased mortality. Thus, toxic effects of EE2 exposures were observable at low density but at high density, density-dependence in body size and mortality overwhelmed the effect of EE2. The results from our study provide insight into the relationship between density and EE2 exposures on fish survival and can be used to adjust population dynamic parameters for more accurate population dynamic estimates.

Keywords: Anthropogenic; Effluents; Wastewater; Fathead minnows; Contaminant; Population

INTRODUCTION

Many aquatic systems face a multitude of stressors due to increased urbanization that include habitat modifications, decreasing stream flows, temperature changes, and chemical inputs from wastewater treatment plants (WWTP) [1]. WWTPs are known to be a major point source of toxicants affecting water quality downstream [2]. Much of the effluent water contains traces of pharmaceuticals that chronically expose local fish populations [3, 4, 5]. Chronic exposures can ultimately affect the growth and survival of individuals among various species of fish and at different functional levels [6]. Thus, understanding the effects of exposure to pharmaceuticals can become important when determining if population level changes are occurring among fish.

Estrogenic drugs, primarily synthetic oral contraceptives, are widely observed in WWTP effluents and prior research has indicated that exposure to 17α-ethinylestradiol (EE2) can cause negative effects to fish populations [7, 8]. Not only do estrogens

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negatively affect the immune and endocrine systems of fishes, but the drugs have also been known to decrease juvenile and embryonic survival, reproduction, and cause the expression of vitellogenin in male fish causing feminization [2]. Specifically, exposures to first-generation fathead minnows (*Pimephales promelas*) decreased the production of eggs, hatch success, and juvenile survival [4]. Exposed adult male fish have been observed to exhibit increased levels of vitellogenin causing a decrease in reproduction success and male survival [4, 9]. Ultimately, reduced vital rates can compromise population dynamics in short-lived species living in wastewater effluent sources containing concentrations of EE2 [4].

Density-dependance is known to regulate animal populations [10]. Interactions between population density and exposure to toxicants have also been known to influence the survival of fish [11, 12, 13]. However, for most fish species there are few studies that investigate how density-dependence interacts with toxicant exposures. Measuring total mortality after toxicant exposures has been previously completed but with low fish densities. In a mesocosm EE2 study, the survival of fathead minnows was not influenced by density, but the overall densities were low (10 fish/mesocosm), and food availability was high [14]. Such studies with low fish densities may bias the results of observed survival because the effect of high density may increase mortality. Although few studies have examined the joint effects of density and toxicants on survival, the interactions that have been studied between density and exposure both support and refute this notion. Exposure of *Daphnia galeeata mendotae* to cadmium, maintained survival rates at high densities whereas others found that exposure of *Capitella sp.* to toxicants at high densities caused reduced survival [12, 15]. In addition, when food availability is altered and fish are exposed to toxicants, decreased survival has been observed at low density and high food availability compared to high density and low food availability, likely as a response to compensation [12, 16]. Such mixed results and complex interactions lead us to believe the effects density may have on aquatic species survival after exposure to a toxicant, such as EE2, will depend on the organism's interaction with the toxicant [4, 11, 13].

In the metropolitan areas of Denver, Colorado, USA, 69%-100% of in stream flow is comprised of wastewater effluent [17, 18]. With increased concern of negative effects from chronic exposures of EE2 in wastewater effluents in Colorado, we chose to expose fathead minnows to EE2. In previous studies, higher mortality was observed among juvenile fathead minnows after the adult fish were exposed to EE2 [4]. However, these previous studies did not control or account for juvenile fathead minnow density. Thus, the purpose of this study was to explore how EE2 concentrations coupled with various fish densities affect body size and survival of fathead minnows.

METHODS

Juvenile fathead minnows were acquired from Aquatic Biosystems, Inc. Fort Collins, Colorado, USA. Fathead minnows were chosen because they are common native Great Plains fish species in Colorado, are ideal model organisms for many of the other native species that are facing effects of wastewater effluents and are small-bodied and easy to keep in a laboratory setting. Fish were randomly distributed into 24 polyethylene mesocosm tanks and were supplied with water from College Lake. The water from the lake was filtered through 100 µm filters and disinfected with ultraviolet light. The mesocosms were filled with approximately 1056 ± 4.4 L of water, aerated with ambient air, covered with 6.25 cm^2 netting, and set at a flow rate of 1 L/min-2 L/min.

Fish were fed a constant amount of concentrated *Artemia nauplii* at 2 mL per day. The A. nauplii were hatched in a conical hatch tube (Aquatic Ecosystems, Apopka, FL, USA) with 1 g L^{-1} in 25 parts per 1000 with constantly aerated sea water (Instant Ocean, Blacksburg, Virginia, USA) and incubated for 24 hours at 26°C-28°C [4].

Experimental factors consisted of three nominal concentrations of EE2 (0 ng/L, 5 ng/L, and 10 ng/L) and 8 different fish densities (20, 40, 80, 160, 320, 640, 1,280, 2,560 fish per mesocosm), resulting in three concentrations per density for a total of 24 outdoor mesocosms. The three nominal concentrations were used to compare our results to those of Schwindt et al. [4] with exception that we did not include a 20 ng/L concentration because of high mortality found in their experiment. Following Schwindt et al. [4] exposure methods, 99% pure 17α-ethynylestradiol was dissolved in HPLC grade methanol and pipetted into the middle of each me- socosm at the three nominal concentrations daily around 1700 hours while flows were stopped. Flows resumed the following morning at 900 hours [4]. A static renewal was determined to simulate a pulsed addition of EE2 typically seen below WWTPs. The control exposure (0 ng/L) included 1 mL of methanol. We did not include a control with only water because of the low methanol concentrations used in the 0 ng/L exposure. The experiment lasted approximately five months. At the end of the experiment, water was drained from each mesocosm, and all remaining fish were collected, counted and lengths and weights were recorded. Survival and body size were examined as a function of starting density and EE2 concentration. We did not collect water samples for water chemistry, only nominal concentrations off EE2 are reported. However, the experimental methods and nominal concentrations are identical to Schwindt et al. [4] thus we assume that actual concentrations are similar.

Statistical Analysis

The experiment used a linear experimental design. The statistical analysis focused on mortality and length at the end of the experiment as response variables. An analysis of covariance (ANCOVA) was used to determine if there were differences in body size (end length) due to estradiol exposure and starting density numbers. The difference was analyzed with EE2 exposure, start density, and their interaction (EE2 exposure × start density) as factors to explain differences in length at the end of the experiment. If there was evidence of a difference in length, then a pairwise comparison with a Tukey's Honest Significant Difference (HSD) adjustment was implemented.

Mortality, defined as the number of dead fish divided by total number of fish at the start, was calculated for each tank at the end of the experiment. To investigate the effects of density on mortality (low density vs. high density) a post hoc regression analysis was used consisting of a two-part modeling approach [19]. Previous studies suggest that low fathead minnow densities have previously been described in the wild as 360 fish per

m^2 and high densities upwards to 1,440 fish per m^2 [20]. Thus, this information combined with the drastic changes in slopes between densities 320 to 640 fish/mesocosm shown in figure 2, to define low densities as less than 320 fish and high densities as greater than 320 fish in our post hoc analysis. First, we used a logistic regression to quantify the difference in mortality due to density. The response is specified by a binary variable 0 if low density (less than or equal to 320 fish) or 1 if high density (greater than 320 fish) with predictor variables of EE2 exposure, starting density and their interaction (EE2 exposure × start density). Chi-squared values were then used to determine if there were statistical differences in mortality based on the predictor variables. Second, we used a beta regression to separately compare the mortality among EE2 exposures, starting density and the interaction for lower density tanks and higher density tanks.

RESULTS

Average fish length at the end of the experiment ranged between 26 mm and 67 mm (**Figure 1**).

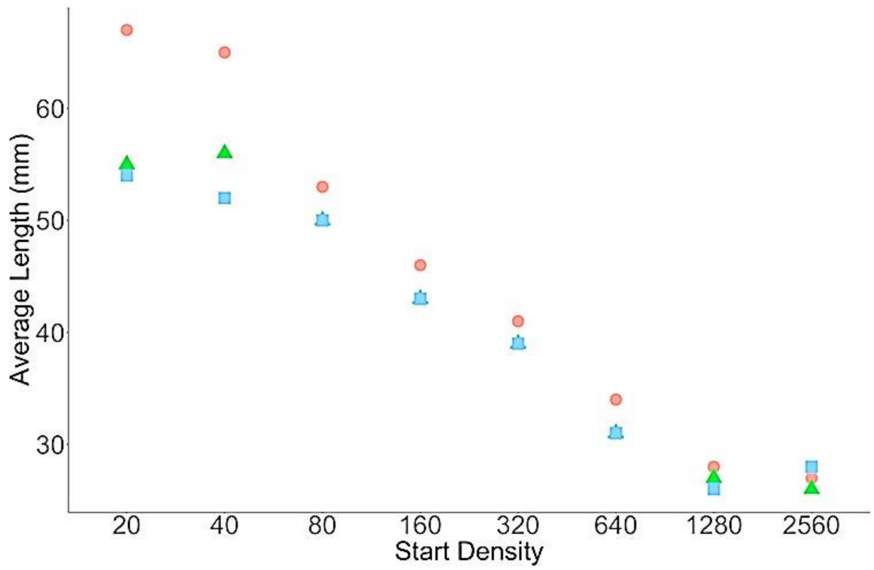

Figure 1: Average length of fathead minnows within each mesocosm by starting density (20 fish/mesocosm to 2,560 fish/mesocosm) and 17α-ethinylestradiol (EE2) exposure (0 ng/L, circles; 5ng/L, triangles; and 10ng/L, squares) at the end of the experiment.

The ANCOVA results indicated that starting density affected body size at the end of the experiment, ($F_{1,18}$=13.44, p-value<0.05) but EE2 treatments did not affect body size ($F_{2,18}$=0.95, p-value=0.40). The post hoc Tukey's HSD showed differences between all but three density pairwise comparisons (160:320 p-value=0.17, 640:1250 p-value=0.12; 640:2560 p-value=0.12). Overall density and body size were correlated with one another and therefore confounded.

Mortality ranged between 0.05 and 0.25 for low densities and increased to as high as 0.87 for high densities (**Figure 2**).

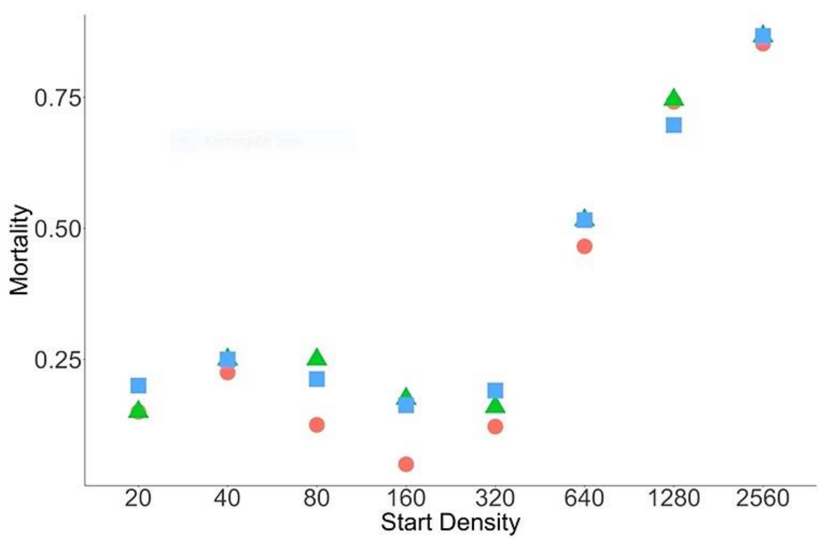

Figure 2: Mortality within each mesocosm by starting density (20 fish/mesocosm to 2,560 fish/mesocosm) and 17α-ethinylestradiol (EE2) exposure (0 ng/L, circles; 5ng/L, triangles; and 10ng/L, squares) at the end of the experiment.

The post hoc regression analysis indicated that there were differences in mortality when comparing low density versus high density (X_1^2=31.76, p-value<0.05). The beta regression for the low-density data points indicate EE2 exposures greater than 0 ng/L increase mortality (z=-7.49, p-value<0.05) and starting density had no effect on mortality (z=-1.72, p-value=0.09). There were no detectable differences in mortality between the 5 ng/L and 10 ng/L EE2 concentrations (z=-0.29, p-value=0.95). The beta regression for the high-density data points resulted in EE2 treatments having no effect on mortality (X_2^2=0.55=0.28, p-value=0.76) and starting density increased mortality (X_1^2=103.19, p-value<0.05). Our results indicate that survival of the fathead minnows is density dependent. The negative effects of EE2 exposure were observed at lower densities, however, were ameliorated by increasing density when density was greater than 320 fish.

DISCUSSION

Effects on reproduction and survival of fathead minnows exposed to EE2 have been studied, but these studies did not control for the effect of juvenile density on juvenile survival [21, 4]. Our study focused on understanding if juvenile density and EE2 exposure influenced body size and mortality among juvenile fathead minnows. Despite concerns that the effects of toxicants may be exacerbated when fish population densities are high, we did not detect an effect of EE2 exposure on mortality at high juvenile density. Juvenile fathead minnow density strongly influenced mortality and body size when densities were higher than 320 fish. When densities were below 320 fish concentrations of EE2 increased mortality compared to controls. Few other studies that assess the effects of toxicants on population dynamics note that toxic effects were masked or potentially ameliorated under high density conditions [16, 22, 23].

Toxicants are capable of prompting disruptions in various biological pathways including behavior and the effect of juvenile fish density on juvenile survival [14, 24, 25, 26]. In ecological modelling, sensitivity analyses have indicated that after EE2 exposures, population growth rate is most sensitive to juvenile survival in fathead minnows [14]. Thus, any additional alteration to juvenile survival might influence the population growth rate due to declining survival of the fish. Density-dependent effects have been observed in unexposed juvenile fathead minnows; [27] however, the influence of density on the effects of a toxicant exposure will likely depend critically on the fish's interaction with the toxicant. At high densities (>320 fish), density-dependent effects may have compensated for toxicant-caused mortality by increasing food availability and decreasing competition, resulting in a greater ability to cope with the onset of stress from a toxicant exposure [23]. It is also possible that the lack of toxicant effects at high density were due to the effect of density overwhelming the ability to detect toxicant effects. At low densities, toxic effects of EE2 exposures were detected but mortality rates never reached as high as in the high-density treatments. Thus, increasing fish density overwhelmed or compensated for toxicant effects on mortality, whereas at low densities juvenile survival was affected by the toxic effects.

When body size is density dependent, a toxicant may exacerbate mortality [16]. However, our results do not support this. At higher densities (>320 fish) body size resulted in smaller fish. According to Barata et al. [16], the smaller fish should have experienced higher mortality due to the toxic effects of EE2, but our data suggest that toxicity did not affect mortality of smaller fish at high densities. However similar to Schwindt et al. [4] the true concentrations of EE2 during the experiment are low- er, not accurately reflecting the actual concentrations during exposures. Thus, we believe our nominal values are similar to those reporting 3.2 ng/L for the 5 ng/L exposure and 5.3 ng/L for the 10 ng/L exposure [4]. It is possible that the differences in actual concentrations may have led to the insignificance of toxicity on body size. Nevertheless, our data indicate that body size is density-dependent and may be an indication of poor food availability due to the number of fish present. Although insignificant, we did visually observe a difference in low 20 and 40 fish densities from the 5 ng/L and 10 ng/L EE2 exposures compared to the controls indicating that at low densities EE2 exposures may decrease body size and should be considered for further investigation.

CONCLUSION

Chronic exposures of estrogenic compounds to fish populations have the potential to be influenced by fish densities through effects on mortality or body size. Understanding the interaction of density with toxicant exposure will allow a more nuanced understanding of population dynamics and management. Our study provides a baseline assessment of the effects of density on body size and survival on fish exposed to estrogens from wastewater effluents.

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CONFLICT OF INTEREST

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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INSTITUTIONAL REVIEW BOARD STATEMENT

The animal study protocol was approved by the Institutional Animal Care and Use Committee Review Board of Colorado State University (protocol number 12-3349A).

DATA AVAILABILITY STATEMENT

The data available in this study are not publicly available but can be available on request from the corresponding author.

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