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Investigation of Histological Effects of 17-α Ethinylestradiol in Fathead Minnows (*Pimephales promelas*)

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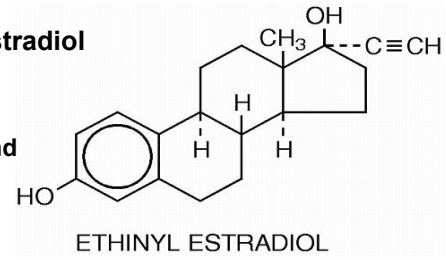

ETHINYL ESTRADIOL

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Abstract

Oral contraceptives are drugs that have been popular for more than 40 years; and many of them contain 17-Alpha Ethinylestradiol (EE2). Women usually absorb 80% of each EE2 dose and excrete the remaining 20%. This unused portion then enters a waste stream that flows into a sewage treatment plant. Recent testing of waste water effluent documented that EE2 exists in surface waters. EE2 is a potent hormone, and several scientific publications have demonstrated it has detrimental effects on aquatic species. Research measured the effects of EE2 on the native Fathead minnow, *Pimephales promelas*, by mimicking a range of drug concentrations found in environments that typically occur in streams and rivers near waste water treatment facilities. We used Fathead minnows because they can survive in a range of clear and turbid waters, and previous research successfully used them. The hypotheses were that male fish would have impaired gonadal development, altered sexual differentiation, and feminization of male reproductive ducts. Juvenile fish from the same cohort were separated into four treatments: control with no estrogen, and three different EE2 concentrations which simulated low, middle, and high field levels previously identified in earlier research. Dissections occurred following six weeks of exposure, and all fish were scored into gonadal development classes based on comparisons against control fish. Male fish significantly had lower adult gonadal development as a result of exposure to EE2 (X^2=8.9, pd0.05). Female fish were not significantly different. Future research includes testing for specific estrogen concentrations, and evidence of VTG synthesis.

Introduction

Most oral contraceptives use 17-α Ethinylestradiol (EE2) as a synthetic replacement for estrogen. When women use these contraceptives they typically absorb 80% of the EE2 dose and waste 20% (Zhang et al. 2007). Waste water treatment plants receive this EE2-containing effluent and remove some organic wastes, but not all of the EE2 (Fent et al., 2006). This treated effluent then reenters the water table via movement from the plant into surface waters. EE2 concentrations in these released effluents have been linked to significant negative histological and reproductive effects on aquatic organisms, including fish (Fent et al., 2006).

Negative effects may include male fish expressing sexual characteristics found in female fish. For example, presence of developing oocytes in males, and abnormal concentrations of sex steroid hormones were found in fish located close to discharge areas of sewage treatment plants that produced measurable EE2 concentrations (Jobling et al., 2006). Responses to EE2 in male fish can cause juveniles to remain sexually immature and appear to be females; potentially leading to loss of species (Liney et al., 2005). Histological analyses of fish exposed to EE2 at varying concentrations resulted in malformed sperm ducts, ovarian cavities in the testes, and the presence of cilia in sperm ducts (Nash et al., 2004). We predicted that EE2 would cause male fathead minnows (*Pimephales promelas*) to have reduced gonadal development thus leading to more juveniles in the older population.

Materials and Methods

Eight glass 37.9 L tanks were placed in a room with a 14 hour day:10 hour dark schedule. A 75.7 L tank housed all minnows at the beginning of the experiment to acclimate them with their environment, as well as to eliminate unhealthy fish. After one day of acclimation, the minnows were separated into the eight tanks, with approximately ten Fathead Minnows being placed into each tank (Fig. 1). Two populations of approximately 100 juvenile fish were purchased because several fish in the first set did not survive transport and acclimation from Fisher Scientific. So a second juvenile population was purchased from the local bait shop.

Prior to fish placement in each tank, and each time water was changed, conditioners were added to deactivate harmful compounds including chlorine and chloramines. Standard aquarium filters were used in order to keep ammonia, nitrate, and nitrite levels normal Fathead minnows typically use rocks for hiding places and breeding. In order to simulate these hiding spots, we placed one clay pot within each aquarium.

Materials and Methods, cont.

After three days of acclimation in the large tank, both populations were distributed among the treatment tanks. Minnows were fed twice daily with standard flake food. Fish were allowed to acclimate for 10 days before treatment began.

Three serial dilutions were made from an EE2 stock solution that contained 70% Ethanol. These dilutions simulated three different EE2 levels found in effluent-containing freshwater rivers and streams (Brian et al. 2005, Liney et al. 2005). Serial dilutions included low, middle, and high contaminations at 5 ng/L, 39 ng/L, and 73 ng/L, respectively. Two control tanks were left untreated. Contamination began on September 30, 2008 and continued for six weeks. The stock solution was stored at -80 C°.

Every week, 50% of each tank's water was changed to maintain cleanliness. Concurrently, EE2 was replenished to original concentrations to adjust for loss from water changes. As the study progressed, minnows that died prematurely were saved for dissection study, but were not used in the final analyses. Any minnows that died were labeled and stored in a deep freezer to minimize the degradation of any EE2 that may be present within their system.

Following the six week contamination period, minnows were euthanized by exposure to 500 mg/l of Benzocaine for 20 minutes. The minnows were then stored by tank number and concentration with the rest of the minnows until dissection.

Dissections of all minnows followed, with data recordings of sex, weight, and length as well as any abnormalities found in the reproductive anatomy. Fish were placed into five age categories: unknown, underdeveloped female, female, underdeveloped male, and male; based upon comparison of standard mature gonadal size and structure.

Data were analyzed using a chi-square test with a 0.05 level of significance. It was expected that if there were no EE2 effects, then all fish should appear as adults.

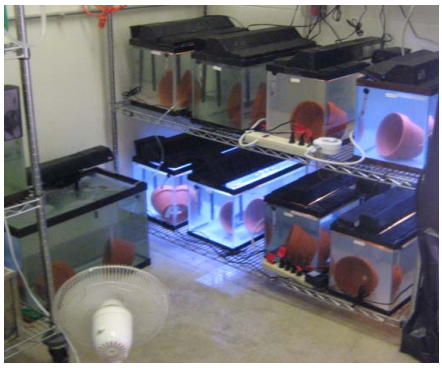

Figure 1. Tank set-up with 75.7L tank on the left. Photo taken by Zachary Barnes.

Results

There was a significant difference between the number of adult male fish compared to the number of underdeveloped male fish (Fig. 2, X^2=8.9, p≤0.05). There was not a significant difference between the number of adult female fish compared to the number of underdeveloped female fish (Fig. 2, X^2=0.4, p≥0.98).

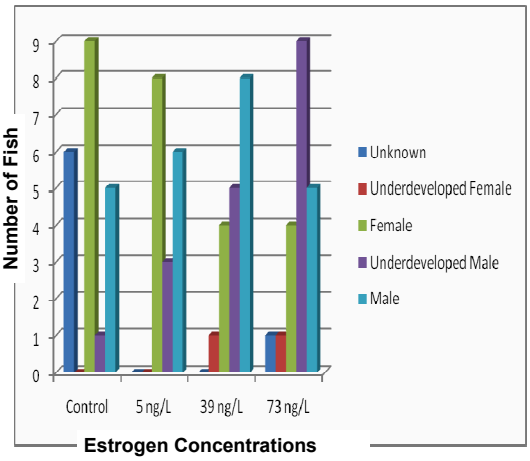

Figure 2. Comparisons of control and treated fish placed in respective age and sex classes. Figure numbers were actual counts (76 total, 27 females, 42 males, 7 were unknown)

Conclusions

Within six weeks, adult male fish had significant gonadal abnormalities when exposed to EE2 contamination.

There was not a significant difference between the number of adult female fish compared to the number of underdeveloped female fish. Many females appeared to have larger than average gonads compared to the controls, but this age/sex category was not part of the original experimental design. Based on these observations, 'overdeveloped females' could be an important age/sex category.

Given that the effects were significant within six weeks, longer studies with even lower concentrations of EE2 should be conducted to determine the threshold of adverse effects on species.

Acknowledgements

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