Inverse Effects On Growth And Development Rates By Means Of Endocrine Disruptors In African Clawed Frog Tadpoles (*Xenopus laevis*)

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Abstract

Previous work on fish, frogs, and salamanders, showed the ability for estrogen (EE2) and anthropogenic endocrine disruptors to skew sex ratios and cause hermaphrodism. This study addressed the effects of estrogens on growth and development rates of African clawed frog tadpoles (*Xenopus laevis*) during their gender determination stages. The effects of estrogen mimics (EM2) have never been studied side-by-side with EE2; I investigated the effects of estradiol β-17 (EE2) and ethynylestradiol α-17 (EM2). Tadpoles were kept individually in containers filled with either EE2 (100 ng/ml), EM2 (100 ng/ml), or dH_2O control. Stage transitions and body sizes were monitored daily over three weeks. Results showed EE2 and EM2 caused faster development rates relative to the control ($p < 0.001$) and slower growth rates relative to the control ($p < 0.005$). While there were no detectable differences between EE2 and EM2 development rates, the growth rate in the mimic treatment was significantly slower than the growth rate in the EE2 treatment ($p < 0.001$). The inverse correlation between development rate and growth rate has implications for the onset of sexual maturity and fecundity. The difference in the growth rates between EE2 and EM2 may be explained by the higher binding affinity of the mimic, which in turn causes the mimic to stay in the body longer than EE2 and may exacerbate the effect on growth rate. How this affects populations of amphibians needs to be explored further.

Introduction

Estrogen (EE2) determines sexual development and behavior while endocrine disruptors, which bind to the same sites as EE2, can have detrimental effects on normal EE2 function (Engell et al., 2005). Anthropogenic EE2 and endocrine disruptors exist at measurable levels in the waters across the world, including Japan, the United States and in Africa (Bennet & Metcalfe, 1998). Fresh and saltwater animals, including fish, frogs and salamanders, are directly affected by measurable levels of EE2 and estrogen mimics (EM2) in the water through increased occurrences of gonadal deformations, unbalanced sex ratios, and decreased ability to reproduce (Clark et al., 1997; Hayes et al., 1999, 2000, 2002; Tollefsen, 2002). Even though there is potential that EE2 or EM2 in the environment might affect humans, it is quite clear from research that they are already affecting aquatic animals (Noriega & Hayes, 2000). Amphibians have been among the hardest struck groups of animals due to their sensitive physiological state and their high exposure to EE2 or EM2 in the environment (Iguchi et al., 2001).

EM2 are entering the water system because so many different common industrial compounds have estrogenic effects (Bayley et al., 1999; Tollefsen 2002). One of the main contributors to the different types of EM2 in the aquatic environment are the byproducts of the plastic making process. There are few regulations on these chemicals and, even if treated, there can be spills of untreated chemicals into the water (Engell et al., 2005). The effects of EM2 have been studied in mammals and fish. A study on the pine vole, a primarily monogamous creature, (Engell et al., 2005) found that females that were injected on a regular basis with the plastic byproduct tended to lose their monogamous tendencies. It was speculated that changes in their brain neural activity were making them act significantly more polygamist compared to the female pine voles who had not been exposed to the plastic byproduct EM2 (Engell et al., 2005). A study by Tollefsen (2002) looked at many different types of EM2 to determine specifically which was causing the problems in fish

(Latonnelle et al., 2002; Tollefsen 2002). Some of the specific ones that were identified were: ethynylestradiol, diethylstilbestrol, genistein and many others that had similar structure to EE2.

The impact of EE2 and EM2 in the environment has been studied extensively; however, animals exposed to EE2 or EM2 have not been compared side by side for possible differences in effects. Additionally how EE2 and EM2 affect early growth and development has not been studied. This study addresses both the comparison of EE2 and EM2 and the effects on early growth and development in the sensitive gender determination stages of *Xenopus laevis*, the African clawed frog,

Methods

The study organism was *Xenopus laevis*. The stages of development are well characterized and with a microscope, it is possible to determine how far a tadpole has developed (Nieuwkoop & Faber, 1956). The control consisted of dH_2O. The EE2 solution was made by dissolving, 0.0230g of estradiol β-17 in 250 mL of 70% ethanol. The EM2 solution was made by dissolving 0.0230 grams of ethynylestradiol α-17 in 250 mL of 70% ethanol. Then 1 ml of either solution was mixed with 1000 ml to achieve a final concentration of 100 ng/mL. Thirty sterile Petri dishes were filled with either 30 mL of the control, EE2, or EM2 for a total sample size of 90 dishes. A permit was acquired from the North Carolina Wildlife Resource Commission; that allowed for the possession of *Xenopus laevis*. The *Xenopus laevis* embryos were ordered from Nasco Supply Co. Tadpoles were randomly assigned to treatments and were placed individually into Petri dishes. Every day for three weeks each Petri dish was cleaned, 30 mL fresh solution were added, and each Petri dish received 250 micro liters of dissolved food/day. Every day, each of the tadpoles was observed so that their stage could be determined (Nieuwkoop & Faber, 1956) and their nose diameter measured. This was done by moving each Petri dish under a photographing microscope and a picture at a set magnification, 40X, was taken of their nose. These photographs were analyzed (Motic 1.3, 2000) by drawing a line from the outer edge of each nostril, and the length

of this line was recorded as 'nose diameter'. Data analysis consisted of calculating descriptive statistics (mean ± standard deviation) of the variables measured. Additionally, confidence intervals were calculated to compare slopes for growth and development rates and an analysis of variance was performed on nose diameter and stage data (JMP INTRO, 2002).

Results

Comparison of developmental rates (Fig. 1), done by statistical analysis of the rates of change (i.e., calculation of the confidence intervals of the slopes), revealed that the slope of the control (0.768) was significantly different from the slope of the EE2 (1.006, $p < .001$) and the slope of the mimic (1.023, $p < .0001$). At the end of the experiment the control animals average stage was 52.3, (± .0666, 1 standard error) the EE2 average stage was at 53.9, (± .0333) and the mimic average stage was 54.1 (± .0577).

Fig. 1. Stage measured versus the stage of the tadpole development. (N = 90). There are three different lines each line representing a different treatment group. One standard error is shown.
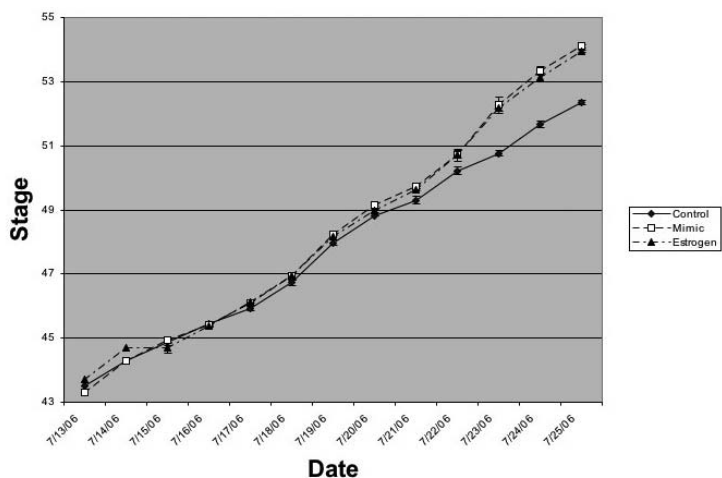
A comparison of stage and nose diameter (a measurement of size) shows an inverse correlation for the estrogen treatments relative to the control (Fig. 2). The nose diameters across the treatments were significantly different (ANOVA 2df, p = 0.0004). The stage data among the treatments were significantly different (ANOVA 2df, p < 0.0001).

A Tukey's mean separation determined which treatment means were different (see Fig. 2 legend for details).

Fig. 2. Comparison of stage and nose diameter by treatment. The left y-axis represents average nose diameter (Ìm). The right y-axis represents average developmental stage (Nieuwkoop & Faber, 1956). Both diameter and stage were measured on the last day of study. One standard error is shown. Letters indicate different means: Nose A, B, C; Stage a, b.
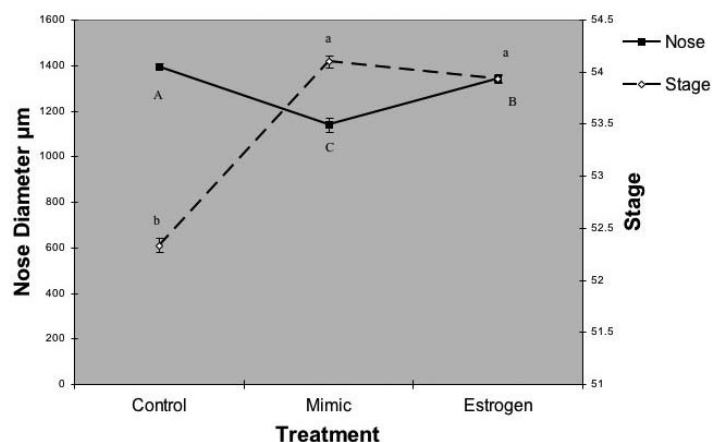
Discussion

There was an inverse relationship between development rate and growth rate across the different treatments (Fig 2). This is surprising because often growth and development are coupled. The control tadpoles developed the slowest, but they grew the fastest. On the other extreme, the EM2 tadpoles grew the slowest but, they were much quicker in their development. These data show that tadpole growth can be stunted by EE2 or even more severely by EM2. On the other hand, both of these chemicals can speed up the rate for development time. The stages between 52-54 are the most important gender determination stages (Nieuwkoop & Faber, 1956) and these are the stages where EE2 and EM2 start to increase development speed (Figure 1). The implication of a shorter development time is that frogs may reach sexual maturity at a younger age and a smaller body size. This suggests that though beyond the scope of this study, frog populations, exposed to EE2 or EM2, may have a greater amount of young, small sexually mature frogs than a non-exposed population.

The primary means by which any estrogenic compound has an effect on the body is by binding to estrogen receptors (Landvatter & Katzenellenbogen, 1982). Estrogen receptors are protein binding sites that can be found throughout the body and have binding sites specific for EE2 to bind to and then cause a specific action, depending where in the body the receptor is. The effect of the EE2 is only felt for as long as the EE2 is bound to the estrogen receptor; because the EM2 has a greater effect than the EE2, it is possible that EM2 may have a longer half life within the estrogen binding sites.

Conclusion

It is clear that EE2 and EM2 greatly sped up the rate of development in *Xenopus laevis*. While the rate of development was being sped up, the growth rate was being slowed down. The inverse correlation between development rate and growth rate has implications for the onset of sexual development and fecundity. The differences between the effects of EE2 and EM2 may be mediated at the estrogen receptors. Estrogen receptors are the highly specialized chemical binding points where EE2 or EM2 bind to have an effect. This study showed how these chemicals affected early developmental stages in tadpoles; now the effect of these chemicals over multiple generations needs to be evaluated. Finally, this study brings to the foreground the need to assess the effects of endocrine disruptors on the earliest stages of growth and development in other aquatic organisms.

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