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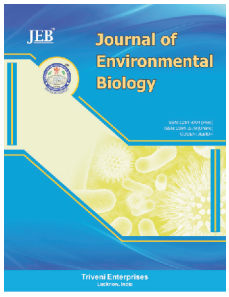

Effects of 17α-ethinylestradiol on embryo survival, hatchability and larval deformities in African catfish, *Clarias gariepinus*

Authors Info

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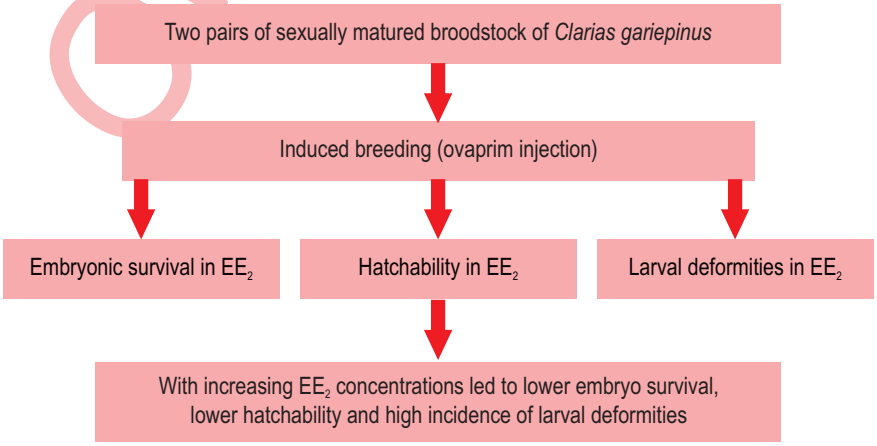
Abstract

Aim : Synthetic estrogens, such as 17α-ethinylestradiol (EE_2), are common in contraceptive pills, and EE_2 levels may be present in various waterways. As such qualitative and quantitative assessment on the impact of EE_2 on aquatic organisms are needed. The main objective of the study was to determine the effect of EE_2 at the environmentally relevant concentrations on embryonic survival, hatchability and larval deformities in African catfish *(Clarias gariepinus)* cultured under laboratory conditions.

Methodology : Two healthy sexually mature males and two gravid female fish weighing 0.8 to 1.0 kg were selected based on the external morphological features. They were then subjected to induced breeding with ovaprim, given at 0.25 b.wt. and 0.5 ml kg^{-1} b.wt. for males and females, respectively. Eight hundred fertilized eggs (embryos) were randomly distributed into four groups with 100 eggs per group in duplicate in respective concentrations of EE_2: control (0 ng l^{-1}), 25 ng l^{-1}, 50 ng l^{-1} and 100 ng l^{-1}, each in 1000 ml glass beakers. Percentage of normal embryonic survival and abnormalities were recorded prior to hatching at 30th hour post fertilization (hpf), while 20 post-hatch larvae were sampled randomly from each group to determine the number of larvae displaying morphological deformities.

Results : At 30th hpf, only 62% of the eggs survived when incubated with 100 ng l^{-1} of EE_2, while the highest survival was observed in the control group (92%). The lowest hatching success rate of 55% was seen in the 100 ng l^{-1} group, which was significantly lower from the embryos exposed to 0 or 25 ng l^{-1}.

Interpretation : The results suggest that EE_2 at 100 ng l^{-1} affected the early life stages of *C. gariepinus*, thus, a closer monitoring on EE_2 discharge into the aquatic environments is essential.



Introduction

Bioassay techniques are keystones for toxicity testing, which helps detect potential threats of either naturally occurring or man-made chemical compounds. In non-mammalian vertebrates, aquatic bioassay provides an excellent tool for this purpose, however, the effect may vary with species and life stages. Compared with the adult stage, early stages of organisms are considered to be the most sensitive to environmental stress (Buikema, 1982; Rasowo, 2007). This is due, in part, to fish embryos and larvae lacking functionally developed gills and kidneys, thus there is increased passive absorption of chemicals from the aquatic environment. Various groups of chemicals are released in to the aquatic ecosystems and, among these, synthetic estrogens such as 17 α-ethinylestradiol (EE_2), in surface waters have been reported to be less than 5 ng l^{-1} (UK Environment Agency, 2002) but higher levels of 17.2 ng l^{-1} (Beck *et al*., 2005), 42 ng l^{-1} (Ternes *et al*., 1999) and 831 ng l^{-1} (Kolpin *et al*., 2002) have also been reported. EE_2 is mostly found in oral contraceptive pills, prescribed at an average daily dose of 30-35 µg per pill (Wise *et al*., 2010), as well as being used in hormone replacement therapies (Desbrow *et al*., 1998; Gutendorf and Westendorf, 2001).

EE_2 is known as a potent synthetic estrogen that also acts as an endocrine disruptor in fish (Kime, 1998). It has strong affinity for the estrogen receptor (Dietrich and Krieger, 2009; Hogan *et al*., 2010) with the ability to concentrate in fish tissue (Thorpe *et al*., 2003; Soares *et al*., 2009). Endocrine disruption has been suggested as the possible cause of reproductive abnormalities for many animal species in the wild. These abnormalities are evident at the early stages of development because some permanent effects may appear, such as various body malformations. Estrogens play an essential role equally in reproduction and somatic cell function, sexual differentiation, ovulation, regulation of mating and breeding, as well as other biological processes such as maintaining mineral and water homeostasis (Fairbrother, 2000).

In the laboratory, at environmentally relevant concentrations, EE_2 causes alterations in the reproductive function of many animals (Jobling *et al.,* 1995; Giesy *et al*., 2000; Fenske *et al.,* 2005). For instance, intibition of spawning in medaka fish, *Oryzia melastigma* exposed to 50-100 ng l^{-1} EE_2 for 14 days (Lee *et al.,* 2014). Chronic exposure of zebrafish *Danio rerio* to 5 ng l^{-1} EE_2 caused a 56% reduction in fecundity with no reported fertilization success (Nash *et al.,* 2004). Microarray analyses of gonads in adult zebrafish exposed to 5 ng l^{-1} EE_2 significantly changed the expression of genes located in the ovaries and testes (Santos *et al*., 2007). Most studies on EE_2 have been focusing on sexually mature adult fish (Van den Belt *et al.,* 2003; Xu *et al.,* 2008). To date, little information is available on the effect of EE_2 on early life stages of fish with most studies focusing on small experimental fish models such as zebrafish (Soares *et al.,* 2009). This study was undertaken to determine the dose-dependent effect of 17α-ethinylestradiol on the early life stages of African catfish *Clarias gariepinus,* one of the most commonly consumed commercial freshwater aquaculture species in South East Asia.

Materials and Methods

Broodstock : Two healthy sexually mature males and two gravid female fish weighing 0.8 to 1.0 kg were selected, based on the external morphological features described by Ayinla *et al.* (1994). Mature male fish was identified by a slightly pointed genital papilla, whereas the females were identified by a swollen abdomen and a reddish swollen vent. The fish were kept in individual tanks and fed *ad libitum* with fish feed (35% crude protein) twice daily (7 am and 5 pm) to satiation based on 5% of the total fish biomass. The broodstock were acclimated in their new environment for at least 3 days at 28 ± 2°C under normal 12 hrs light: 12 hrs dark photoperiodic regime, with water pH ranging from 6.1 to 6.8 and dissolved oxygen 6.8 to 7.5 mg l^{-1}.

The females were administered with ovaprim at 0.50 ml kg^{-1} b.wt. whereas the males were administered with 0.25 ml kg^{-1} b.wt. All ovaprim-induced breeding was administered at 22:00 hrs. Briefly, the fish was covered with wet towel, and Ovaprim was injected intramuscularly above the lateral line towards the dorsal section and pointed towards the ventral side. After withdrawing the needle, the fish was finger-rubbed to avoid the backflow of the injected fluid. The injected fish was returned to their respective tanks. Approximately, 10 hrs after the administration of ovaprim, the pre-weighed females were examined for their ovulatory response. The release of eggs through the genital pore following gentle pressure on the abdomen was considered as a commencement of ovulation and the eggs were stripped into fertilization trays. Testes were then removed from the males, and sperm was squeezed into a clean petri dish and mixed with the eggs.

Embryonic development and hatchability : The stripped eggs were pooled and fertilized with the pooled sperm suspension diluted with saline solution. After 2 min of gentle stirring, the fertilized eggs were washed several times with fresh water to remove any excess milt. The eggs were spread into a single layer on suspended nylon mesh for incubation. One hour post fertilization, the unfertilized eggs were removed carefully from the incubation tank. A sample of 800 fertilized eggs (embryos) were randomly distributed into four groups with 100 eggs per group in duplicate: control (0 ng l^{-1}), 25 ng l^{-1}, 50 ng l^{-1} and 100 ng l^{-1}, each in 1,000 ml glass beakers. Aeration was provided at a rate of 30 fine bubbles per minute. A stock solution of EE_2 (>98% purity, Sigma-Aldrich, USA) was freshly prepared by dissolving the reagent in ethanol. The stock solution was diluted to their respective concentrations with dechlorinated tap water containing 0.01% ethanol for the control and treatment groups (Lee *et al.,* 2014).

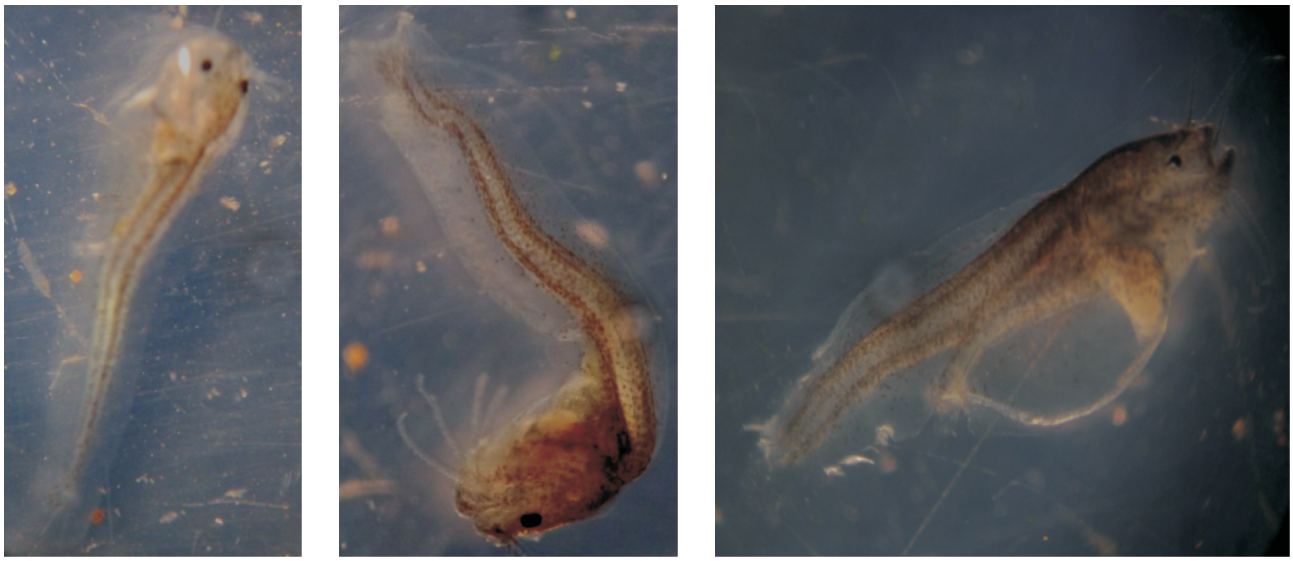


Fig. 1 : Normally developed type of deformities in larvae of *Clarias gariepinus* (far left), larvae with scoliosis (middle) and with yolk sac edema and gaped jaw (far right)

Water temperature, pH and dissolved oxygen levels were measured every 8 hrs during the experimental period. The percentage of embryo survival and abnormalities were recorded prior to hatching at 30th hour post fertilization (hpf). In addition, the hatching rate was also recorded and expressed as follows:

$$\frac{\text{No. of eggs incubated - No. of unhatched eggs}}{\text{No. of eggs incubated}} \times 100$$

Larval deformities : Following hatching, 20 post-hatch larvae were sampled randomly from each group, and the number of larvae displaying morphological deformities were recorded and photographed.

Statistical analyses : Statistical analyses were performed with SPSS version 20. Shapiro–Wilk's test was used to check the assumption of normality, and Levene's test was used to check the assumption of homogeneity of variance. The influence of EE_2 on survival rate, hatching rate and percentage of post-hatch larvae abnormalities among the groups were analyzed by one way. Percentage data was ANOVA transformed prior to analyses. The data was presented as mean ±SD and significance level was considered at $p < 0.05$.

Results and Discussion

The perusal of data showed a dose-dependent relationship between the tested EE_2 concentrations and the parameters measured. At 30th hpf, only 62% of the eggs survived when incubated with 100 ng l^{-1} of EE_2, which was significantly lower compared with the other groups (Table 1). The highest survival was observed in the control group at 92%, as well as hatchability. Both hatchability and survival in the control group, were, not significantly different from 25 and 50 ng l^{-1} groups. The lowest hatching success rate of 55% was seen in the 100 ng l^{-1} group, which was significantly different from embryos exposed to 0 or 25 ng l^{-1}.

In addition, the number of newly hatched larvae with abnormalities increased with the increasing concentrations of EE_2. The incidence of deformity was 5% at 100 ng l^{-1}, and was significantly higher ($p<0.05$) than the other groups (Table 2). Scoliosis, yolk sac edema and gaped jaws were among the deformities observed (Fig.1). The tested water quality parameters were similar among the treatments and were within the acceptable range for *C. gariepinus* eggs (Table 3), therefore, the cause for these findings was due to EE_2 concentrations.

One of the key active compounds found in birth control pills is 17α-ethinylestradiol (EE_2), which is a synthetic estrogen and potent endocrine modulator (Desbrow *et al*., 1998; Langston *et al*., 2005). This has been shown to exert a range of estrogenic effects in fish at environmentally relevant concentrations (Purdom *et al*., 1994; Thorpe *et al*., 2003).

In this study, 100 ng l^{-1} EE_2 reduced the embryo survival, hatching success rate but increased the percentage of larval deformities, compared with 25 and 50 ng l^{-1}. As such 100 ng l^{-1} may not be the teratogenic threshold for African catfish. However, the threshold could be more than 50 ng l^{-1} but less than 100 ng l^{-1}, which were not covered in the present study. A similar observation was also reported by Versonnen and Janssen (2004), where zebrafish exposed to 100 ng l^{-1} EE_2 showed significant effects on the survival, hatching and growth. Previous studies have also revealed a significant concentration-dependence in hatching

Table 1: Percentage of embryonic survival after 30 days and hatchability of *Clarias gariepinus* exposed to increasing concentrations of EE_2

EE_2 (ng l^{-1})	Embryo survival	Hatchability
0	92.0 ± 3.0[a]	70.0 ± 2.0[a]
25	89.0 ± 4.0[a]	65.0 ± 2.0[a]
50	82.0 ± 5.0[a]	63.0 ± 2.0[ab]
100	62.0 ± 9.0[b]	55.0 ± 2.0[b]

*Different superscript letters indicate significant differences ($p<0.05$); Values are mean ± SD.

Table 2 : Percentage of newly-hatched larvae with deformities in *Clarias gariepinus* exposed to increasing concentrations of EE_2

EE_2 (ng l^{-1})	Larval deformities (%)
0	1.0 ± 0.0[a]
25	1.5 ± 0.7[a]
50	2.5 ± 0.7[a]
100	5.0 ± 2.8[b]

*Different superscript letters indicate significant differences ($p<0.05$); Values are mean ± SD.

Table 3 : Minimum and maxium temperature (°C), pH and dissolved oxygen (DO in mg l^{-1}) throughout the study

Parameters	Minimum	Maximum
Temperature	26.5	28.9
pH	6.3	6.9
DO	6.9	7.5

success to sheepshead minnow (Zillioux *et al*., 2001) and mummichog (Peters *et al*., 2010) exposed to 200 and 100 ng l^{-1} EE_2, respectively. In contrast, no differences were observed in survival, weight or length of hatched medaka exposed for 2 months to 1, 10 and 100 ng l^{-1} EE_2 (Scholz and Gutzeit, 2000). However, a later study showed the fertilization rate and hatchability in Japanese medaka decreased at 500 ng l^{-1} EE_2 (Tilton *et al*., 2005). These findings may best be explained by interspecies differences. For instance, expression of estrogen receptors ERα and ERβ in fish gonads and liver have been shown to differ between the species (Socorro *et al*., 2000; Tchoudakova *et al*., 1999). In addition, EE_2 has been demonstrated to enhance the expression of the CYP19A2 gene in zebrafish (Kazeto *et al*., 2004) and medaka (Scholz and Gutzeit, 2000), which codes for an aromatase enzyme that mediates the conversion of androgen to estrogen. CYP19A2 has been detected in the brain of developing embryo of killifish, *Fundulus heteroclitus* (Dong and Willet, 2008).

In the present study, as the EE_2 concentration increased, the number of larval abnormalities increased, and was significantly higher at the highest concentrations used (100 ng l^{-1}). Several types of deformities were observed in this study *i.e.,* fused vertebrae, scoliosis, gaped jaw and yolk sac edema. According to Onuoha and Nwadukwe (1990), spinal flexures of the larvae appeared to be a common response to various environmental stresses during ontogenic development. In this study, observation showed disturbances in the swimming behaviour of the larvae with deformities in addition to appearing less energetic.

The results of the present study, revealed that a relatively short exposure of *C. gariepinus* embryos and larvae to 100 ng l^{-1} EE_2 adversely affected their embryonic development, hence reduced survival and hatchability rates, as well as larval deformities. Similar to the results of a study previously conducted on roach, *Rutilus rutilus,* by the UK Environment Agency (2008), the results of this study suggested that EE_2 at 100 ng l^{-1} affected the early life stages of *C. gariepinus*, thus, warrants closer monitoring on EE_2 discharge into the aquatic environments.

It is concluded that EE_2 at 100 ng l^{-1} poses a potential teratogenic risk to the early life stages of *C. gariepinus*. Further investigations on the mechanisms of EE_2 on early life stages of *C. gariepinus* are required, particularly at the molecular level which can be integrated to be informative from an ecological point of view.

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