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Reproductive toxicity in *Xenopus tropicalis* after developmental exposure to environmental concentrations of ethynylestradiol

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ABSTRACT

Reproductive disorders in wildlife and humans have been linked to developmental exposure to endocrine disrupting chemicals. In frog tadpoles, environmental concentrations of ethynylestradiol (EE_2) disrupt gonadal differentiation which results in female-biased sex ratios at metamorphosis indicating sex-reversal of genotypic males. It is not known if developmental exposure to estrogens results in reduced reproductive success in amphibians. The objective of this work was to investigate if exposure to environmentally relevant concentrations of EE_2 during sex differentiation impairs reproductive organ development, fertility, and sexual behavior in adult frogs. A specific aim was to evaluate if testicular structure and function was affected in males that were not sex-reversed. *Xenopus tropicalis* tadpoles were exposed until metamorphosis to 6, 60, and 600 pM EE_2. Eight months after metamorphosis, reproductive organ morphology and fertility were evaluated. Larval EE_2-exposure caused an increased proportion of phenotypic females indicating that sex-reversal of genotypic males is persistent. Sex-reversal was implied at concentrations as low as 6 pM (1.8 ng/l), which is comparable to levels observed in the environment. EE_2-exposed males that were not sex-reversed had a significantly reduced fertilization rate compared with control males. Histological evaluation revealed that EE_2-exposed males had a reduced amount of spermatozoa in the testis. Among frogs with ovaries there was a significantly higher percentage that lacked oviducts in the group exposed to 600 pM EE_2 compared with control females. No effect of EE_2 on sexual behavior was noted. The results indicate that reproduction in wild frogs might be impaired by estrogenic environmental pollutants. Similarities between the present effects and those reported in fish, birds and mammals after developmental exposure to estrogens suggest that *X. tropicalis* is a promising animal model for research on developmental reproductive toxicity.

1. Introduction

Many environmental pollutants can interfere with development and function of the endocrine system and have in some cases been linked to wildlife and human reproductive disorders (Helle et al., 1976; Guillette et al., 1994; Toppari et al., 1996; Toft et al., 2003; Olesen et al., 2007). Several endocrine disrupting chemicals, such as ethynylestradiol (EE_2), bisphenol A, alkylphenols, and *o*,*p*'-DDT, have estrogenic activity and can thereby disrupt estrogen-dependent processes such as gonadal differentiation. In wild roach (*Rutilus rutilus*) high incidences of hermaphrodites have been observed in UK rivers polluted with natural and synthetic estrogens (estradiol, estrone, and EE_2) from sewage effluents (Desbrow et al., 1998; Jobling et al., 1998). These intersex fish were reported to have reduced milt production, sperm quality and fertilization success compared to normal male fish (Jobling et al., 2002). Amphibian populations have declined dramatically world wide during the last decades (Griffiths and Beebee, 1992; Houlahan et al., 2000; Alford et al., 2001; Stuart et al., 2004) and in many cases the causes are unknown (Stuart et al., 2004). One of the suggested reasons for this decline is environmental pollution (Gibbons et al., 2000; Gardner, 2001; Beebee and Griffiths, 2005). Observations of high incidences of frogs with testicular oocytes and intersex gonads in wild populations of leopard frogs and cricket frogs in the U.S. have been suggested to result from exposure to environmental pollutants (Hayes et al., 2003; Reeder et al., 2005).

It is known that larval exposure to estrogenic compounds disrupt gonadal differentiation which results in female-biased sex ratios in amphibians (Chang and Witschi, 1956; Richards and Nace,

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1978; Kloas et al., 1999; Mackenzie et al., 2003; Pettersson et al., 2006; Pettersson and Berg, 2007). Studies of long-term effects on the reproductive system in frogs following larval exposure to environmental pollutants are scarce. Larval exposure to the pharmaceutical and environmental estrogen EE_2 at concentrations higher than those found in the environment induces persistent sex-reversal of male *Xenopus tropicalis* frogs (Pettersson et al., 2006). Some of the EE_2-exposed frogs with ovaries lacked oviducts making them sterile (Pettersson et al., 2006). In order to evaluate the risk of impaired reproduction in wild frogs as a result of environmental pollution, there is a need to investigate effects on fertility after exposure to environmental concentrations during larval development.

EE_2 was used in the present study both as a model compound to explore estrogen-induced developmental toxicity and as an environmental pollutant. Concentrations of EE_2 ranging from 7 to 420 pM (125 ng/l) have been found in the canals and the lagoon outside of Venice, Italy (Pojana et al., 2004). In rivers downstream sewage treatment plants in the UK, Germany and the Netherlands maximum concentrations of, respectively, 51 pM EE_2 (15 ng/l), 17 pM (5.1 ng/l) and 15 pM (4.3 ng/l) have been detected (Aherne and Briggs, 1989; Belfroid et al., 1999; Ternes et al., 1999; Kuch and Ballschmiter, 2001). In the U.S., Kolpin et al. (2002a,b) detected concentrations of EE_2 up to 920 pM (273 ng/l), and a median concentration of 320 pM EE_2 (94 ng/l) in rivers downstream sewage treatment plants.

We have previously shown that larval exposure to environmentally relevant concentrations of EE_2 (60 and 90 pM, respectively) cause female-biased sex ratios, implying sex-reversal of genotypic males, in juvenile West-African clawed frogs (*X. tropicalis*) and European common frogs (*Rana temporaria*) (Pettersson and Berg, 2007). The objective of the present study was to investigate if larval exposure to 6, 60, and 600 pM (pmol/l) EE_2 impairs reproductive organ development, fertility, and sexual behavior in adult frogs. A specific aim was to evaluate if testicular structure and function was affected in males that were not sex-reversed. *X. tropicalis* was used as a model species because of its short generation time (about 6 months) compared with other amphibian species (1–3 years) (Witschi, 1929a,b; Ryan, 1953; Hirsch et al., 2002) which makes it suitable for life-cycle studies. The exposure period, from hatching until metamorphosis, was chosen because gonadal differentiation occurs in *X. tropicalis* during this time (Pettersson and Berg, 2007; Takase and Iguchi, 2007). Hormone receptors play an important role in mediating steroid hormone actions. It has been shown that estrogen receptor α and β mRNA is expressed in brain, liver, and gonad/kidney in *X. tropicalis* during gonadal differentiation (Takase and Iguchi, 2007). Sex organ differentiation starts during the larval period in most frog species (Lofts, 1974). As most amphibians pass through an aquatic larval stage they can be exposed to waterborne environmental pollutants during this critical period of reproductive development. The timing and route of exposure in the present study represent, therefore, an ecologically relevant exposure scenario for both terrestrial and aquatic frog species.

2. Materials and methods

2.1. Animals and exposure

Adult *X. tropicalis* (Xenopus 1, Dexter, MI, USA) were mated following treatment with human chorionic gonadotropin (hCG). Tadpoles from four pairs of frogs were exposed to EE_2 (Sigma–Aldrich, St. Louis, MO, USA) from Nieuwkoop and Faber (NF) developmental stage 47–48 (Nieuwkoop and Faber, 1956), until complete metamorphosis (NF stage 66) as described in Pettersson and Berg (2007). The exposure started 4–5 days after hatching and lasted for about 2 months. In short, two replicate exposure tanks were used for each concentration including the control. Chemical analyses of the EE_2-concentrations in the exposure tanks were performed by an accredited commercial analytical laboratory (Analycen Nordic AB, Lidköping, Sweden). The mean measured EE_2-concentrations in the aquaria (measurements made on day 1 and 32 of the experiment) were 5.2 and 6.2 pM (1.5 and 1.8 ng/l) in the lowest, 59 and 62 pM (17 and 18 ng/l) in the middle and 600 and 610 pM (178 and 181 ng/l) in the highest concentration group (Pettersson and Berg, 2007). EE_2 was dissolved in acetone and all tanks, including control tanks, had an acetone concentration of 0.0001%. The water flow rate was 60 ml/min and the flow rate of EE_2 and control acetone were 50 μl/min. After metamorphosis, 55–63 randomly selected frogs per concentration were kept for 8 months in a flow-through system (60 ml/min) using nine parts deionised water and one part copper-free tap water at a density of one frog per litre. The animals were kept in a water temperature of 26 ± 1 °C and a 12:12-h light:dark cycle. Tadpoles were fed Sera micron (Sera, Heinsberg, Germany) and metamorphosed frogs were fed Aquatic nature tropical fishfood – Excel S (Aquatic Nature, Roeselare, Belgium) and Hikari Staple (Kyorin, Kamihata, Himeji, Japan). They were treated weekly with nifurpirinol (0.24 mg/l) (Aquamor, Aquarium Münster, Telgete, Germany), malachite green (40 ng/l), and formalin (4 ng/l) (Tetra Pond Desafin, TetraWerke, Melle, Germany) to prevent infectious disease outbreaks. Levels of ammonia and nitrite were measured monthly using standard tests from Merck (Damstadt, Germany). The study was approved by the Local Ethics Committee for Research on Animals.

2.2. Fertility studies

For the fertility studies the frogs had their sex determined based on secondary sex characteristics including body shape, occurrence of nuptial pads in males, and enlarged cloaca in females. Females are normally larger and more pear-shaped than males. Ten or eleven randomly chosen females from each concentration group, 22 control males and all EE_2-exposed males were evaluated for breeding success. These frogs were mated with 11-month-old unexposed frogs that had not been mated before. Mating was induced by injecting hCG into the dorsal lymph sac in both males and females. The first injection of 20 IU (international units) hCG (in 100 μl of 0.9% NaCl) was given 24 h prior to mating. Just before breeding a second dose of 100 IU hCG (in 100 μl of 0.9% NaCl) was injected. For the mating one female and one male were placed in a mating aquarium containing 4 l of water (nine parts deionised water and one part copper-free tap water). The mating couples were observed once every 45th min during at least 6 h. After 6 h had elapsed and the males had released amplexus the frogs were killed. Amplexus success (frequency of frogs that went into amplexus), the time from the last hCG injection until amplexus, and the duration of amplexus were recorded. The total number of eggs in the mating aquaria was counted or, in case of more than a thousand eggs, approximated. One day after mating, the number of developing embryos was counted (approximated if more than a thousand). Developing embryos were distinguished from unfertilized eggs by an oval shape and greater size. The fertilization rate was calculated as the number of developing embryos/number of laid eggs per aquarium × 100. The frogs were given a code number so that all evaluations of reproductive performance were made without knowledge of what exposure the frog had been subjected to.

2.3. Dissection of reproductive organs

The frogs were anesthetized in 0.5% benzocaine (Sigma–Aldrich, St. Louis, MO, USA) dissolved in 70% ethanol. They were weighed,