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Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: II. Effects on gonad histomorphology

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Abstract

A number of man-made chemicals has been shown to mimic endogenous hormones and to induce alterations of reproductive physiology in wild populations. Of particular importance are compounds that mimic estrogens and androgens (and their antagonists), because of their central role in reproductive function. In this study, male and female adult South African clawed toads (*Xenopus laevis)* were exposed to ethinylestradiol (EE2), tamoxifen (TAM), methyldihydrotestosterone (MDHT) and flutamide (FLU) as (anti)estrogenic and (anti)androgenic model compounds, respectively, at a concentration of 10^{-8} M, and to water from the river Lambro (LAM), a contaminated watercourse from Northern Italy. Potential disrupting effects on reproduction were studied by histological analyses of gonads. The strongest adverse effects were observed in EE2 and LAM exposed males, e.g. tubule mean diameter reduction, spermatogenic nest breakdown and interlobular wall thickening. In both groups, the occurrence of small oocytes within the seminiferous tubules was observed. In TAM and MDHT exposed females slight oocyte atresia and occurrence of spermatogenic nests were observed. In contrast to previous studies addressing the alteration of molecular biomarkers in the same experimental setup, histological analyses of gonads were very sensitive and indicated an adverse effect of water from Lambro River on reproductive physiology of *X. laevis*.

Keywords: Amphibians; Endocrine disrupting chemicals; Gonad morphology; Lambro river; Xenopus laevis

1. Introduction

Many environmental contaminants either of natural (e.g. phytoestrogens) or anthropogenic (e.g. industrial by-products) origin are described to affect reproductive biology of vertebrates by mimicking or antagonizing the action of hormones. The environmental presence of these substances, called Endocrine Disrupting Chemicals (EDCs), have been only rarely related to reproductive disturbances in wild mammals (Facemire et al., 1995; Harding et al., 1999; Vos et al., 2000; Fossi and Marsili, 2003), birds (Fry and Toone, 1981; Giesy et al., 1994), reptiles (Guillette et al., 1994; Guillette and Iguchi, 2003) and fish (Jobling et al., 1998, 2002; Van Aerle et al., 2001). Nevertheless EDCs are found almost everywhere in the environment and they mainly accumulate in surface waters and sediments. For this reason, permanent or occasional inhabitants like fish and amphibians face an increased risk of being harmed by EDCs. Amongst aquatic species, amphibians represent a unique target in the environment. They are either important predator or prey components of aquatic and terrestrial ecosystems, because of

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their aquatic way of living during breeding, their sensitive phase of sexual differentiation, and their terrestrial adult life phase (Carey and Bryant, 1995; Fort et al., 2004). Several studies indicate that, amongst a variety of physical and chemical stressors, EDCs may contribute to the general decline of amphibians (Weller and Green, 1997; Houlahan et al., 2000; Stuart et al., 2004). In fact, in response to EDC a number of reproductive abnormalities (Bögi et al., 2002, 2003; Hayes et al., 2002a,b, 2003; Mosconi et al., 2002; Pickford et al., 2003), increased mortality and embryo malformation (Burkhart et al., 1998; Fort et al., 2001); altered gonad differentiation and development and sex ratio (Kloas et al., 1999; Ohtani et al., 2000, 2001; Qin et al., 2003; MacKenzie et al., 2003), impaired spermatogenesis (Lee and Veeramachaneni, 2005), gonadal dysgenesis (Fort et al., 2004; Hecker et al., 2005), hermaphroditism (Reeder et al., 1998; Hayes et al., 2003), feminization (Palmer and Palmer, 1995; Hayes and Menendez, 1999; Levy et al., 2004a; Kloas and Lutz, 2006) and inhibition of ovarian steroidogenesis (Pickford and Morris, 2003) have been described in amphibians. Effects of EDC on the reproductive system of animals are often mediated via steroid receptors. Thereby chemicals may act as agonists or antagonists of the estrogen or androgen receptor.

The South African clawed toad *Xenopus laevis* is an amphibian species consolidated as an *in vitro* (Kloas et al., 1999; Fort et al., 2002; Lutz et al., 2005) and *in vivo* (Bögi et al., 2002, Kloas, 2002; Levy et al., 2004b; Lutz and Kloas, 1999; Urbatzka et al., 2006, 2007b) model for the screening of EDCs affecting reproductive biology, thyroid system (Opitz et al., 2005, 2006) and neural development (Bevan et al., 2000). In this study *X. laevis* was used as an *in vivo* model to study the effects of model EDCs with (anti)estrogenic and (anti)androgenic modes of action on gonad morphology. Adult *X. laevis* were exposed to ethinylestradiol (EE2) as estrogenic compound, tamoxifen (TAM) as anti-estrogenic compound, methyldihydrotestosterone (MDHT) as androgenic compound and flutamide (FLU) as anti-androgenic compound in a four week exposure at a concentration of 10^{-8} M. Moreover, aiming to increase the knowledge about the environmental hazard, a group of *X. laevis* was exposed to Lambro river water, a well known polluted source of EDs of Northern Italy (Viganò et al., 1994, 1998; Fattore et al., 2002) able to induce intersexuality in cyprinids (Viganò et al., 2001). More recently, estrogenic, androgenic and anti-androgenic activities have been demonstrated *in vitro* both in water and sediment fractions from the Lambro river (Urbatzka et al., 2007b). The present paper focuses attention on establishing morphological endpoints for the detection of effects of EDCs on the gonads of *X. laevis*, such as testis length and width, seminiferous tubule diameter, relative abundance of spermatogenic nests and occurrence of oocytes in males and oocyte stage distribution and occurrence of oocyte atresia in females.

2. Material and methods

2.1. In vivo exposure

Adult *Xenopus laevis* (3–4 years old) were taken from the breeding stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin. The frogs were fed twice a week and kept on a 12:12 h light : dark cycle.

For the exposure, adult male and female *X. laevis* were transferred to aerated 10 L tanks containing reconstituted tap water using distilled water supplemented with 2.5 g marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany). *X. laevis* were exposed to tamoxifen (TAM), ethinylestradiol (EE2), flutamide (FLU) and methyldihydrotestosterone (MDHT), chosen as (anti)estrogenic and (anti)androgenic compounds, respectively, and to Lambro river water. Chemicals were dissolved in ethanol (Roth, Karlsruhe, Germany) and a solvent control (0.001% ethanol) was included in the experimental design of the study. The treatment lasted 4 week s and consisted of 8 females and 8 males per compound equally distributed on two 10 L tanks containing 4 females and 4 males, respectively. The test concentration of the chemicals was 10^{-8} M. Rearing water and chemicals were renewed completely every Monday, Wednesday and Friday. During exposure, *X. laevis* were fed once a week with commercial fish diet (Fisch-Fit, Interquell, Wehringen, Germany) and water temperature was adjusted to 22±1 °C.

2.2. Chemicals and Lambro sampling

All test chemicals (EE2, TAM, MDHT, FLU) were purchased from Sigma (Taufkirchen, Germany). The river Lambro is a tributary to the river Po in Northern Italy, contaminated with agricultural run-off as well as domestic and industrial waste

Table 1
Stages from *X. laevis* male germ cells development (adapted from Kalt, 1976)

Stage	General appearance
Primary spermatogonia	Large cell, often in excess of 20 μm, surrounded by somatic cells. Large convoluted, euchromatic nucleus with prominent nucleoli
Secondary spermatogonia	Cluster of cells still organized in nests. Round nucleus with heterochromatic patches
Leptotene primary spermatocytes	Large round nucleus with a thin, slightly condensed chromatin and a single–double nucleolus
Zygotene primary spermatocytes	Intermediate features, due to partial synapsis and condensation of sister chromatids within the nucleus. The nucleus shows regions mildly condensed
Pachytene primary spermatocytes	The nuclear outline remains round and a single nucleolus is present. Synapting chromatids that appear as distinct thickened fibers
Diplotene primary spermatocytes	Very short stage characterized by separation and definition of chromatid bivalents
Secondary spermatocytes	Smaller nucleus with condensed chromatin
Spermatids	Dense nucleus from round to elliptical shape in the three different sequential developmental sub-stages in concomitance with the develop of acrosoma vesicle
Spermatozoa	Characteristic elongated shape, densely packing in Sertoli cell cytoplasm