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Mate calling behavior of male South African clawed frogs (*Xenopus laevis*) is suppressed by the antiandrogenic endocrine disrupting compound flutamide

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ABSTRACT

Several environmental pollutants have been identified as antiandrogenic endocrine disrupting chemicals (EDC), with flutamide (FLU) being a model compound for this type of action. Despite impacts of EDC interfering with sexual differentiation and reproduction in amphibians, established information about suggested effects on sexual behavior is still lacking. In this study adult male *Xenopus laevis* were injected with human chorionic gonadotropin (hCG) to initiate mate calling behavior. After one day hCG-stimulated frogs were treated via aqueous exposure over three days without and with FLU at concentrations of 10^{-8} and 10^{-6} M in comparison to untreated frogs. Androgen controlled mate calling behavior was recorded during the 12 h dark period. At the end of exposure circulating levels of testosterone (T) and 17β-estradiol (E2) were determined and furthermore gene expression was measured concerning reproductive biomarkers such as hypophysial luteinizing hormone (LH), follicle-stimulating hormone (FSH), testicular aromatase (ARO), 5α reductase type 1 (SRD5α1) and 5α reductase type 2 (SRD5α2). Both concentrations of FLU caused a significant decrease in calling activity starting at the second day of exposure. HCG injected positive controls had elevated levels of T compared to negative control frogs while in parallel treatment with FLU did not affect significantly the hCG elevated sex steroid levels. Furthermore, hCG treatment led to significantly decreased levels of gene expression for ARO and SRD5α2 but no impacts were detected on LH, FSH or SRD5α1 mRNA levels compared to negative controls. In summary, the behavioral parameter mate calling is the most sensitive biomarker detecting antiandrogenic modes of action in this challenge-experiment indicating that this non-invasive method could markedly contribute for sensitive assessment of antiandrogenic EDC.

1. Introduction

In recent years intensive research evolved concerning the emerging field of endocrine disrupting chemicals (EDC) which effects adversely reproductive biology and development of vertebrates. Concerning sexual differentiation and reproductive biology of amphibians four modes of action (MOA) of EDC, (anti)estrogenic and (anti)androgenic ones, are known (Kloas, 2002; Kloas and Lutz, 2006). EDC can activate/block the androgen (AR) or estrogen receptor (ER) or affect sex steroid synthesis and thus they can be defined as chemicals with (anti)androgenic or (anti)estrogenic MOA. The interaction with the specific receptor results in an altered binding activity to promoter sequences of receptor-regulated genes which in turn causes different rates of gene expression affecting biological functions like steroidogenesis (Govoroun et al., 2001; Thibaut and Porte, 2004), gonadal development, sexual differentiation (Miyata et al., 1999; Kloas, 2002; Akatsuka, et al., 2004; Lee et al., 2004) or secondary sex characteristics (Baatrup and Junge, 2001; Bayley et al., 2003; van Wyk et al., 2003; Katsiadaki et al., 2006; Kloas and Lutz, 2006). *Xenopus laevis* has been established as suitable model-organism to study the effects of EDC. Exposure of EDC or exogenous steroids caused feminization (Kloas et al., 1999; Bögi et al., 2002; Levy et al., 2004) neutralization or masculinization in *X. laevis* tadpoles (Kloas, 2002; Kloas and Lutz, 2006). In adult animals the hepatic biomarker vitellogenin (VTG) is upregulated by estrogens and downregulated by antiestrogens (Palmer and Palmer, 1995; Kloas et al., 1999; Urbatzka et al., 2007b). Transthyretin and transferrin are regulated down by estrogenic compounds and up by antiestrogenic compounds (Urbatzka et al., 2007a). Different patterns exist concerning hypophysial luteinizing hormone (LH) and follicle-stimulating hormone (FSH) expression as a result to (anti)estrogenic and

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(anti)androgenic exposure (Urbatzka et al., 2006). In the teleostean stickleback the spiggin egg protein has shown to be a specific antiandrogenic biomarker (Katsiadaki et al., 2006; Kloas et al., 2009). Despite those findings a highly sensitive and easily accessible biomarker in amphibians is still missing.

Like in all vertebrates reproductive biology is under the regulatory control of the hypothalamic–pituitary–gonadal (HPG) axis. Mate calling activity is an important factor of amphibian reproductive behavior in male *X. laevis* and is activated by elevating levels of the endogenous sexual-steroids testosterone (T) and dihydrotestosterone (DHT). Frogs treated with human chorionic gonadotropin (hCG) showed increased sexual behavior (Russell, 1954; Hutchinson and Poynton, 1963; Kelley and Pfaff, 1976; Wetzel and Kelley, 1983). Injection of T, DHT or hCG is accompanied by increased calling activity.

The aim of the present study was to show whether the antiandrogenic model compound flutamide (FLU) can affect hCG stimulated mate calling activity in male *X. laevis* and thus to establish an new non-invasive method to detect antiandrogenic MOA in water samples. As the HPG-axis is mainly responsible for the regulation of reproductive biology, biomarkers representing the different stages of the HPG-axis were analyzed to validate behavioral findings in a comprehensive way. Expression of hypophysial LH and FSH and testicular aromatase (ARO), 5α reductase type 1 (SRD5α1) and type 2 (SRD5α2) was analyzed. In addition plasma concentrations of the endogenous sexual steroids T and 17β-estradiol (E2) were measured.

2. Materials and methods

2.1. Animals and in vivo exposure

Adult male *X. laevis* (2–4 years) were taken from the breeding stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin. Mean body weight was 7.7 ± 2.1 g. The frogs were fed twice a week and kept with a light:dark cycle of 12 h:12 h. Three days before the start of exposure individual frogs were transferred into 54 L tanks (60 × 30 × 30 cm) containing 27 L tap water and kept visually and acoustically isolated from each other. Recording in the dark period started 24 h before the animals received an injection into the dorsal lymphsac containing 300 i.U. hCG (dissolved in 150 μl deionized H_2O) or 150 μl deionized H_2O as negative control. Furthermore antiandrogenic treated animals were exposed to FLU dissolved in 500 μl ethanol resulting in final concentrations of 10^{-8} M and 10^{-6} M, respectively. Solvent control group was included in experimental design for both controls the sham treated as well as the hCG injected control. Treatment solutions including control groups were refreshed daily and total exposure time to FLU was 72 h. Animals were not fed during testing period. Water temperature was maintained at 20.2 ± 0.8 °C.

2.2. Sampling

At the end of exposure period animals were anesthetized and sacrificed. Blood-samples were taken from the dorsal arteries and centrifuged immediately at 2000*g* for 1 min. Fifty microliters of plasma was transferred into 1.5 ml microcentrifuge tubes and 1.2 ml ethanol p.a. was added, thoroughly mixed and then centrifuged for 2 min. The supernatant containing the extracted steroids was transferred into glass vials and after evaporation of liquid phase at room temperature samples were closed and stored at −20 °C for later analyses of sex steroid concentrations. To determine gene expression of biomarkers testes and pituitaries were collected and stored in RNA-Later (Qiagen) at −20 °C.

2.3. Sex steroid determination

Plasma levels of E2 and T were determined by using commercially available enzyme linked immunosorbent assays (ELISAs (Cayman Chemicals, Ann Arbour, MI, USA). The assays were performed according to manufacturer's protocol. For determination samples were re-dissolved and diluted using 5% ethanol p.a. (1:10 for E2, T).

2.4. RNA-isolation and reverse transcription

Extraction of total RNA was performed by using RNA extraction kits (RNeasy Mini Kit, Qiagen, Germany) according to the instructions of the manufacturer and included on-column DNAse (Qiagen) digestion. Reverse transcriptase reaction (RT) was performed with 1 μg total RNA. For RT 7.5 pM poly(dT) primer (Biometra, Göttingen, Germany), 10 mM dNTP (Qbiogene, Heidelberg, Germany) and 10 U AMV reverse transcriptase (Finnzymes, Finland) were applied in a 30 μl reaction volume.

2.5. PCR

Amplifications of specific cDNA for LHβ, FSHβ, ARO, Srd5α1, Srd5α2 and elongation factor-1α (EF1α) genes of *X. laevis* were carried out by using semi-quantitative RT-PCR. For amplification of ARO, Srd5α1 and Srd5α2 genes cDNA was diluted 1:10. Three microliters of either diluted or undiluted cDNA was mixed in a 25 μl reaction volume with 10 mM dNTP's (Qbiogene), 1.5 mM $MgCl_2$, 10 pM forward and reverse primer (sequences according to Urbatzka et al., 2006, 2007b) and 1 U of Taq Polymerase (Qbiogene). For PCR of Srd5α2, Taq Polymerase (Platinum, Invitrogen) and reduced $MgCl_2$ concentration (1 mM) was used. Annealing temperatures and cycle numbers are shown in Table 1. Determinations were always performed in the linear range. Semi-quantitative analysis including gel electrophoresis of samples with 1.5% agarose gel containing ethidium bromide and densitometric measurement was performed using an image analyzer (Gel Doc 2000, Bio-Rad, München, Germany) (Urbatzka et al., 2006). Densitometric values obtained were normalized by values obtained for the housekeeping gene EF-1α (Krieg et al., 1989). Levels of EF-1α remained unchanged by all treatments.

2.6. Mate calling activity

From the day before hCG-injection till the end of exposure period, the calling activities of male *X. laevis* were recorded in the 12 h dark period (DP). To record calling activity hydrophones (SQ 26, Sensor Technology, Collingwood, Canada) were used in combination with a multichannel soundcard (Delta 1010 LT, M-Audio, Öhringen, Germany). Hydrophones were fixed to ensure center position and to minimize echo interference. Tanks were placed individually and acoustically isolated from each other to avoid cross-talk events. For automated recording a bandpass of 2.5 kHz (range: 1–3.5 kHz) was chosen as Wetzel and Kelley (1983)

Table 1
Annealing temperatures and cycle numbers of used primers.

Gene	Annealing temperature (°C)	PCR cycles
EF1α, elongation factor 1α	59	18
ARO, aromatase mRNA	59	32
Srd5α1, steroid 5-α-reductase, polypeptide 1 mRNA	55	26
Srd5α2, steroid 5-α-reductase, polypeptide 2 mRNA	55	29
FSHβ, follicle-stimulating hormone β-subunit mRNA	63	30
LHβ, luteinizing hormone β-subunit mRNA	62	25