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Aqueous leaf extracts display endocrine activities *in vitro* and disrupt sexual differentiation of male *Xenopus laevis* tadpoles *in vivo*

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ABSTRACT

The occurrence of natural substances acting as endocrine disrupting compounds (EDC) in the environment is to date poorly understood. Therefore, (anti)androgenic and (anti)estrogenic activities of three different aqueous leaf extracts (beech, reed and oak) were analyzed *in vitro* using yeast androgen and estrogen screen. The most potent extract was selected for *in vivo* exposure of *Xenopus laevis* tadpoles to analyze the potential effects on development and reproductive biology of amphibians. Tadpoles were exposed from stage 48 to stage 66 (end of metamorphosis) to aqueous oak leaf extracts covering natural occurring environmental concentrations of dissolved organic carbon. Gene expression analyses of selected genes of the hypothalamus–pituitary–gonad and of the hypothalamus–pituitary–thyroid axis as well as histological investigation of gonads and thyroid glands were used to evaluate endocrine disrupting effects on the reproductive biology and development. Female tadpoles remained unaffected by the exposure whereas males showed severe significant histological alterations of testes at the two highest oak leaf extract concentrations demonstrated by the occurrence of lacunae and oogonia. In addition, a significant elevation of luteinizing hormone β mRNA expression with increasing extract concentration in male tadpoles indicates an involvement of hypothalamus–pituitary–gonad axis mainly via antiandrogenic activity. These results suggest that antiandrogenic EDC of oak leaf extract are responsible for inducing the observed effects in male tadpoles. The present study demonstrates for the first time that in surface waters, natural occurring oak leaf compounds at environmentally relevant concentrations display antiandrogenic activities and have considerable effects on the endocrine system of anurans affecting sexual differentiation of male tadpoles.

1. Introduction

Different chemicals have been identified to interfere with the endocrine system of several animal groups and are therefore defined as endocrine disrupting compounds (EDC). Reproductive biology and the thyroid system of different animal groups are the most frequently studied targets of EDC. The adverse effects on reproductive biology of vertebrates related to the exposure to EDC have been attracted a lot of attention over the past decades. Widespread are the known disturbance effects on the gonadal development (Kiparissis et al., 2003; MacKenzie et al., 2003), spermatogenesis (Bayley et al., 2003), induction of sex reversal (Villalpando and Merchant-Larios, 1990; Gimeno et al., 1998), hermaphroditism (Reeder et al., 1998) and feminization (Palmer and Palmer, 1995; Kloas et al., 2009). Furthermore, alterations of the thyroid system caused by EDC are also of concern, because thyroid hormones mediate a variety of physiological processes, like regulation of growth and metabolism (Brown et al., 2004) or amphibian (Tietge et al., 2005) and fish (Manzon et al., 1998) metamorphosis.

Despite the wide range of recent research on environmental EDC of anthropogenic origin, the knowledge about EDC occurring naturally is marginal. In the aquatic environment (anti)thyroidal (Theodorakis et al., 2006), estrogenic (van der Linden et al., 2008), antiestrogenic (Murk et al., 2002), androgenic (Thomas et al., 2002) and antiandrogenic activities (Urbatzka et al., 2007a) have been derived from anthropogenic and natural sources. Pulp and paper mill effluents were shown to possess androgenic activities (Svenson and Allard, 2004) that could be partly explained as microbial degradation of phytosterols to androgens (Jenkins et al., 2004). Beside this, the masculinization of fish populations caused by pulp and paper mill effluents was described for Florida (Parks et al., 2001) and Sweden (Larsson and Forlin, 2002).

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Synthetic humic substances caused feminization in amphibians (Lutz et al., 2005), which pointed to natural organic sources as possible new players in the area of endocrine disruption. The degradation of plant material, in particular leaves, could potentially result in the natural occurrence of EDC in the aquatic environment considering that endocrine activities have been already described for different plant extracts (Baker et al., 1998; Ong and Tan, 2007). Fallen leaves constitute up to 30% of the organic matter input in the aquatic system (Meyer et al., 1998). So, due to senescence (cell membrane integrity collapse with subsequent major loss of leaves) a dominant amount of organic matter is introduced into the water column at least once per year (Wetzel, 2001). The whole dissolved organic carbon (DOC) concentrations in most oligotrophic freshwater ecosystems normally ranges between 1 and 100 mg/L DOC, but also 300 mg/L DOC have been reported (Blodau et al., 2004). Up to now, no effort has been undertaken to characterize endocrine activities of natural aqueous leaf extracts as they occur in the aquatic environment or to assess their potential impact on reproductive biology and thyroid system of vertebrates. Therefore, the yeast androgen screen assay (YAS) and the yeast estrogen screen assay (YES) were applied to investigate endocrine effects of selected aqueous leaf extracts. The YES and YAS are suitable to investigate agonists as well as antagonists of the estrogen and the androgen receptor, respectively (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998). Three plant species were chosen in the present study representing one of the main autochthonous and allochthonous sources of DOC in European aquatic ecosystems. *Phragmites australis* is a worldwide distributed, dominant plant species in many aquatic ecosystems of temperate regions and is able to form huge reed-beds along shallow lakes (Hollis and Jones, 1991; Wetzel, 2001). Oak and beech trees constitute a significant proportion in deciduous and mixed forests in water catchments and lose their leaves seasonally.

After endocrine characterization of the aqueous leaf extracts *in vitro*, the most potent extract was chosen for conducting a long-term *in vivo* exposure using the well established amphibian model organism *Xenopus laevis* (Opitz et al., 2005; Urbatzka et al., 2007b; Das et al., 2009) to detect effects on the development triggered by the thyroid system and reproductive biology. Reproduction of vertebrates is mainly regulated by the hypothalamus–pituitary–gonad axis (HPG). Key hormones of the gonadal development are the gonadotropic hormones, luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). The gonadotropic hormones regulate the gonads in terms of synthesis and secretion of the sex steroids, androgens and estrogens, which subsequently control target cells and cause negative feedbacks on the hypothalamus and the pituitary for regulating hormone homeostasis (Kloas and Lutz, 2006; Kloas et al., 2009). While the gonadal development is regulated by the HPG, the postembryonal differentiation (metamorphosis) is triggered by the hypothalamus–pituitary–thyroid axis. The synthesis and secretion of the thyroid hormones (TH) is stimulated by the pituitarian thyroid-stimulating hormone (TSH). In peripheral tissues TH effects are mediated by binding to specific nuclear thyroid receptors, TRα and Trβ. During metamorphosis the expression of TRα is rather constitutive, whereas expression of Trβ is more variable and closely correlated with changes in circulating TH.

For the *in vivo* investigation *X. laevis* tadpoles were exposed to the chosen aqueous extract from NF stage 48 (Nieuwkoop and Faber, 1994) until the end of metamorphosis. Survival rate, time to complete metamorphosis, weight and whole body length were recorded and sexual differentiation of gonads was investigated gross morphologically and histologically. In addition, gene expression of the key players of the HPG axis, LHβ and FSHβ in brain, and selected steroidogenic genes in the gonads were analyzed to study a potential alteration of different endpoints of the HPG axis. Furthermore, the potential impact on thyroid system was investigated by assessing mRNA levels of TSH and TRβ which are established molecular endpoints for (anti)thyroidal EDC and additional thyroid histopathology of animals taken after 21 days of exposition (Opitz et al., 2006).

2. Materials and methods

2.1. Chemicals

Methyldihydrotestosterone (MDHT), ethinylestradiol (EE2), tamoxifen (TAM), flutamide (FLU), and all chemicals necessary for the media preparation except chlorophenolred–galactoside (CPRG) (Roche Diagnostics, Mannheim, Germany) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Ethanol (EtOH, 99.9%, Roth, Karlsruhe, Germany) was used as solvent for the standard chemicals.

2.2. Preparation and characterization of natural organic matter (NOM) extracts

The aqueous leaf extracts were prepared according to Kamara and Pflugmacher (2007). Leaves from standing dead *P. australis* plants were collected from the littoral zone of Lake Müggelsee in autumn 2006. Fallen dead oak (*Quercus robur*) and beech (*Fagus sylvatica*) leaves from top litter layer were collected within the catchments area of Lake Müggelsee in the same period. After 5 days of air drying the leaves were homogenized using a homogenizer mill (1094 Tecator). 450 g of the received material was added to 2 L of medium containing deionized water, $CaCl_2$ (0.2 g L^{-1}), $NaHCO_3$ (0.103 g L^{-1}) and sea-salt (0.1 g L^{-1}) in separate flasks and stirred for 24 h at room temperature. The resulting mixture was centrifuged (L-60 Ultracentrifuge, Beckman LL-TB-003A) at 20,000*g* for 10 min at 4 °C to remove solid materials. The supernatant was sterile filtered using 0.2 μm cellulose-acetate membrane filter (Sartorius AG, Germany). The amount of DOC of the extracts was analyzed by liquid chromatography organic carbon detection (LC-OCD) as described previously by Huber and Frimmel (1996).

2.3. Assay for the screening of androgenic activity

The recombinant YAS was kindly provided by Prof. Sumpter, Brunel University, UK. All media used for the assay were prepared according to the original protocol (Sohoni and Sumpter, 1998). Briefly, the assay was designed to detect androgen induced expression of the enzyme β-galactosidase by yeast cells. Yeast cells constitutively express the human androgen receptor hAR, which was stably integrated into the genome. The yeast also contains expression plasmids, carrying the androgen responsive elements (ARE) regulating the expression of the reporter gene lacZ (encoding the β-galactosidase). Consequently, if an active ligand (in this case an androgen acting or mimicking substance) binds to the receptor this leads to a conformational change of the receptor necessary for binding of the receptor/ligand dimer to the ARE. Accordingly the β-galactosidase is synthesized and secreted into the medium. After addition of the specific substrate CPRG (yellow) to the medium, CPRG is cleaved by the β-galactosidase into chlorophenolred (red) and galactose.

The assay was performed as described by Urbatzka et al. (2007a): yeast (125 μl) from the yeast stock stored at −20 °C were added to the growth medium and grown on an orbital shaker for about 24 h at 28 °C until the absorption of 1 at 620 nm was achieved. The yeast culture was diluted to an absorption of 0.1 with fresh growth medium and 150 μl were seeded into 96-well microtiter plates that were previously prepared with a dilution