Environmental signaling: synthetic humic substances act as xeno-estrogen on Xenopus laevis

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Introduction

Humic substances are large, complex, organic molecules, which are the main component of dissolved organic matter (DOM) in aquatic ecosystems and with concentrations between 1 and 100 mg/l exceeding the organic matter of all other compounds including biota (Thurman, 1985). Occasionally even more, for instance: Australian wetlands up to 300 mg/L DOC (Chambers et al. 2004), Brazilian coastal lagoons 160–200 mg/L DOC (Graneli et al., 1998). In contrast to outdated paradigms that consider HS high-moelecular weighted, refractory or even inert, recent findings show that water soluble HS are relatively small by molecular weight (Hatcher et al. 2004). Hence, it is not surprising that HS are taken up by aquatic organisms (Steinberg et al., 2003). Once taken up, they may alter growth and reproduction of the nematode *Caenorhabditis elegans* (Höss et al., 2001) and increase the hatching rate, improve the somal constitution, and impact the sex ratio in the swordtail *Xiphophorus helleri* (Meinelt et al., 2004). To date, nothing is known about the role of HS in mediating endocrine disruption of amphibian reproduction. Since previous studies with the nematode *C. elegans* were carried out with natural HS isolates, a diffuse contamination by xeno- or phyto-estrogens cannot be excluded. In subsequent studies, the synthetic HS1500 was applied, which is not contaminated with xeno- or phyto-estrogens.

For studies of the potential effects of HS on sexual differentiation, the South African clawed frog *Xenopus laevis* provides an excellent model organism. The molecular biology of *X. laevis* is well known, and, in contrast to higher vertebrates, the sex ratio in amphibians can be influenced by external factors. Consequently, *X. laevis* is a comprehensive model with which to study potential reproductive effects of endocrine disrupters (ED) *in vivo* and *in vitro* (Kloas et al., 1999; Lutz et al., 1999). Investigation of the expression of specific biomarkers underlying hormonal control, delivers valuable signs of endocrine activity, for example estradiol specifically elevates transcription of mRNAs and translation of the estrogen receptor (Tata, 1987). Furthermore, *in vivo* exposure to endocrine-active substances during larval development impacts sexual differentiation of amphibians and leads to subsequent shifts in sex ratios of the animals (Kloas et al., 1999; Kloas, 2002).

The present study aims to identify HS-mediated effects on reproduction of *X. laevis,* by determining the effects on sexual differentiation during larval development and by monitoring the ER-mRNA expression as an estrogenic biomarker *in vivo*.

Material and Methods

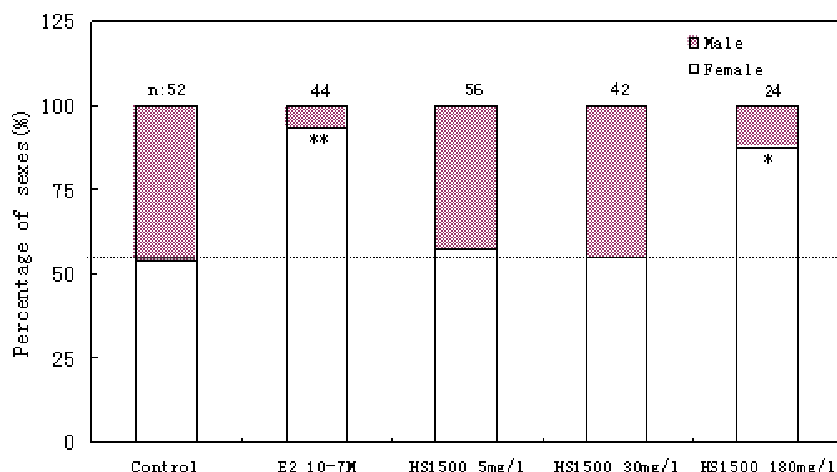
Humic Material HS1500, characterized by dominant aromatic and quinoide structures with a mean molecular mass of 1.5 kDa (Sopar Pharma GmbH, Mannheim, Germany), was selected as a representative HS.

***In Vivo* determination of effects of HS1500 on sexual differentiation** Exposure in vivo was carried out at the developmental stage 40/42 (2-3 days after hatching), using 10-L glass aquaria in a flow-through system. A total of 30 tadpoles were randomly put in each aquarium. The experimental set up divided the tadpoles into 5 groups consisting of a solvent control, the positive control 17ß-estradiol (E2, 10^{-7} M), and the HS1500 groups at nominal concentrations of 5, 10, 30, 60, 90, 180 mg/L, in replicates. During the exposure, the water temperature was controlled at 22 ± 2 °C and the light-dark rhythm was 12:12 (L: D), the test water solution was changed every other day and the tadpoles were fed daily with commercial food pellets. When most of the animals had completed metamorphosis, they were killed and the gonads were assayed for determination of sexual differentiation a binocular with 40-fold magnification (Kloas et al., 1999). Livers were carefully removed and snap frozen immediately in liquid nitrogen for further processing.

Determination of ER-mRNA by semi-quantitative RT-PCR For semiquantitative analysis of ER levels as estrogenic biomarkers, the RT-PCR technique was used. Therefore, Total RNA was isolated from liver samples of Xenopus juveniles, transcribed enzymatically into cDNA by reverse transcription and amplified using polymerase chain reaction. To ensure comparability of results, expression of elongation factor 1a as an internal standard was

determined for each sample (for a detailed description of the method, see Kloas et al., 1999).

Data Analysis Statistical analysis was performed using one-way ANOVA, followed by Dunnet's test. Data were tested for normality and homogeneity of variance using the software package SPSS/PC^{TM}, version 10.0 (SPSS Inc., Chicago, IL, USA).



Results and Discussion

Figure 1 Sexual differentiation of *X. laevis* larvae exposed to HS1500 and E2 10^{-7}M. The estrogen 17ß-estradiol (E_2) serves as a positive control. * indicates significance at $p<0.05$ level, ** significance at $p<0.01$ level; one-way ANOVA correlation analysis.

Sexual Differentiation Exposure to HS1500 at 180 mg/L HS significantly increased the percentage of female phenotypes to 87.5%. A similar significant feminization was obtained in tadpoles exposed to E2 10^{-7} M, that was included as a positive control. Furthermore a dose dependent increase in females at the higher concentrations of HS1500 at 90 and 180 mg/L were found (Fig.1). The results confirm the potential of HS1500 to act as a potent feminization agent. Consequently, the sex ratio of *X. laevis* can be used as a phenomenological parameter by which to evaluate reproductive effects of HS in general. However, the strong feminizing effects *in vivo* suggest additional modes of action such as anti-androgenic ones.

ER-mRNA expression as an estrogenic biomarker *in vivo* Compared to the control detection of mRNA-levels of ER using semi-quantitative RT-PCR revealed a significant increase of ER-mRNA levels in the livers of *X. laevis* females, exposed to 30 mg/L HS1500 whereas 5-mg/L HS1500 exposures did not differ from the control. Comparing the effects of E2$10^{-7}$M and 180 mg/L HS1500 exposures, ER-mRNA expression is similarly elevated in the liver of *X. laevis* females, suggesting a dose response of HS1500 for the estrogenic biomarker ER-mRNA (Fig. 2). HS1500 showed clear significant feminization phenomena as indicated by *in vivo* treatment increasing female phenotypes. However, due to a variety of functional chemical groups in the HS1500, it cannot be excluded that, in addition to potential estrogenic effects, further modes of action might appear, such as anti-androgenic ones which may also induce feminization *in vivo.*

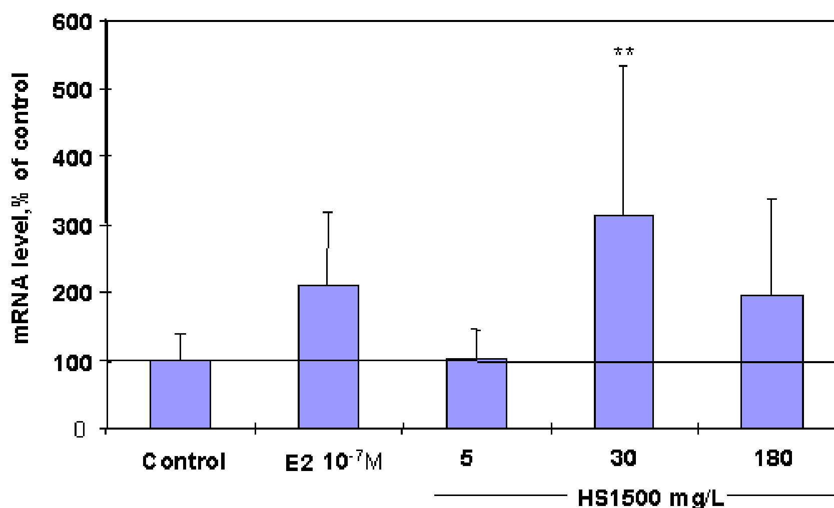


Figure 2 Levels of ER-mRNA in the liver of *X. laevis* females treated with E2 10^{-7}M and HS1500 at different concentrations (n = 6). The bars are means ± SD, ** indicates significance at p<0.01 levels.

Hence, the general statement seems justified: exposure of aquatic vertebrates, such as fish or amphibians, to HS, may result in a feminization.One mode of action appears to be the estrogenic pathway, since increased ER-mRNA levels could be identified. The observed effects are most likely due to the structural units of HS1500 themselves, rather than contamination by xeno- or phyto-estrogens.

References

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