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# Acute and chronic effects of testosterone and 4-hydroxyandrostenedione to the crustacean *Daphnia magna* ☆

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ABSTRACT

Steroid compounds have been globally detected in surface waters. The ecological impacts of these biologically active chemicals are largely unknown. Toxicity of testosterone and 4-hydroxyandrostenedione was assessed for the freshwater cladoceran *Daphnia magna*. Acute toxicity tests showed that 6.20 mg $L^{-1}$ of testosterone, the highest concentration tested, did not have effect on the daphnids, whereas 4-hydroxyandrostenedione had an $EC_{50}$ of 4.26 mg $L^{-1}$. Chronic toxicity tests were carried out using survival, body length, fecundity, and fertility as endpoints. Long-term testosterone exposure reduced *D. magna* fecundity and fertility at concentrations ranging from 0.31 to 2.48 mg $L^{-1}$. The significant decrease in fecundity was associated with an increase in aborted eggs. Long-term 4-hydroxyandrostenedione exposure at 0.84 mg $L^{-1}$ increased the mortality of the neonates. The chronic toxicity effects were observed at concentrations higher than the measured environmental concentrations of these compounds. Nevertheless, the reproductive impairment of the daphnids is likely to occur at environmental levels as an ultimate response to long-term exposure.



## 1. Introduction

The presence of toxic agents in the ecosystem has increased in recent years, especially in aquatic environments. Some chemical products that are released into the environment interfere with the endocrine system of organisms by decreasing sperm count, inducing hormone-sensitive carcinomas (e.g. female breast cancer, testicular, and prostate cancer) in humans, and by causing reproductive abnormalities, feminization of fish and a decrease in the reproductive rate of birds and other wildlife (Colborn, 1995; Guillette and Craine, 2000; Stalschmidtallner et al., 1997; Tyler et al., 1998; Sumpter, 1998).

Anthropogenic chemicals, including natural and synthetic steroid hormones, phytoestrogens, pesticides, surfactants, polychlorinated biphenyls, and pharmaceuticals compounds have been found to have endocrine-disrupting properties (Falconer et al., 2006).

The attention in the field of endocrine-disruption has been focused to the estrogenic effects of chemicals (Folmar et al., 2002; Petrović et al., 2004). More recently, the androgenic and anti-androgenic activities of chemicals have been described in association with paper and pulp mill effluents (Svenson and Allard, 2004), rivers (Thomas et al., 2002), and effluents from wastewater treatment plants (Blankvoort et al., 2005). While androgenic activity in rivers may be a partial result of microbial degradation of phytosterols to progesterone and then to androgens (Jenkins et al., 2004), anti-androgenic activity is derived from anthropogenic chemicals (Sumpter, 2005). Naturally occurring reproductive hormones such as 17$\beta$-estradiol, testosterone, and their metabolites are commonly detected in sewage treatment works (STW) effluents (Desbrow et al., 1998). Kolodziej et al. (2004) reported that dairy wastewater, aquaculture effluents, and even spawning fish as sources of steroids hormones (testosterone, androstenedione, estrone) in aquatic environment.

The estrogenic and androgenic steroid hormones are excreted by vertebrate organisms in the free form or as conjugates (Panter et al., 1999). They are chemically very stable, highly lipophilic and poorly soluble in water (Table 1) hence their biological properties will have a strong impact on non-target species (Henschel et al., 1997).

Testosterone (17$\beta$-hydroxy-4-androstene-3-one), a $C_{19}$ steroid (Table 1), is the most potent naturally secreted androgen in vertebrates and can be used in endocrine therapy as well as being

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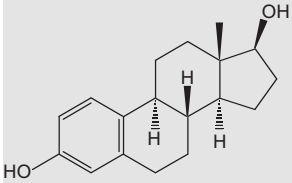
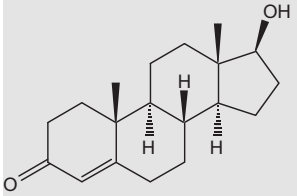
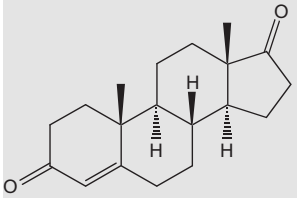
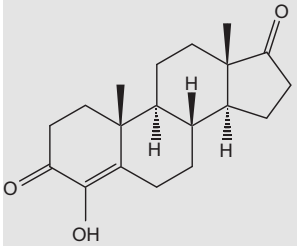
**Table 1**
Molecular structures and physicochemical properties of steroid hormones

| Steroid hormone | Molecular weight ($g\,mol^{-1}$)[a] | Water solubility at 20 °C ($mg\,L^{-1}$) | Log $K_{ow}$ | Melting point (°C)[a] |
|---|---|---|---|---|
| 17$\beta$-Estradiol[b]  | 272.4 | 3.9–13.3 | 3.1–4.0<br>3.784[c] | 171 |
| Testosterone[d]  | 288.4 | 10–25 | 3.2<br>3.219[c] | 155 |
| 4-Androstene-3,17-dione[e]  | 286.4 | 37–41 | 2.817[c]<br>NA[f] | 173 |
| 4-Hydroxy-4-androstene-3,17-dione[a]  | 302.4 | Practically insoluble | 2.7433[c]<br>NA[f] | 196–205 |

[a] The Index de Merck, 12 ed Merck & CO. Inc., NJ, USA.
[b] Hanselman et al. (2003).
[c] Log $K_{ow}$ calculated by software CS ChemOffice 6.0, ChemDraw Ultra 6.0, *Cambridgesoft.com*, 2000.
[d] Lintelmann et al. (2003) and Lee et al. (2003).
[e] Lee et al. (2003).
[f] No data available.

a prohibited substance in human sport (Cowan and Kicman, 1997). The 4-hydroxyandrostenedione (4-OHA; Formestane; Table 1) is a derived compound of androstenedione that can be formed from testosterone by a synthetic pathway of androgens in vertebrates (Recanatini et al., 2002). This compound inhibits the enzyme aromatase, which is responsible for the conversion of androgens to oestrogens, and by this mechanism is used in the treatment of breast cancer (Brodie et al., 1977; Lonning, 1998). The aromatase inhibitors (e.g. 4-OHA) are steroid compounds used clinically and are an androgen alternative used in aquaculture for the production of monosex Nile Tilapia (Lee et al., 2006) or sterile populations of fishes (Piferrer, 2001; Devlin and Nagahama, 2002). Although the concentrations in aquatic environment are relatively low ($ng\,L^{-1}$) the high dosages of exogenous steroids used in aquaculture for manipulation of sex differentiation on in fish (Devlin and Nagahama, 2002), become potentially significant because aquaculture effluents are often discharged to receiving waters with little or no treatment and the concentrations of these compounds exhibit considerable temporal and spatial variation (Kolodziej et al., 2004).

Thus, the aim of this study was to evaluate the aquatic toxicity of testosterone and 4-OHA, using *Daphnia magna*, a freshwater zooplankton. Daphnids are routinely used in aquatic toxicology bioassays because of their rapid reproduction, sensitivity to their chemical environment, and their critical role in freshwater ecosystems (Baird et al., 1989). In aquatic food webs daphnids play an important role intermediating between primary producers and fish, and their life-history changes can trigger community or ecosystem-level responses.

The present study assessed the toxic effects of testosterone and 4-OHA on acute and sub-lethal responses of *D. magna* using survival, growth, moulting frequency, fecundity and fertility as endpoints.

## 2. Materials and methods

### 2.1. Organism and culture conditions

*D. magna* (clone A *sensu*, Baird et al., 1989) obtained from laboratory stock cultures was used throughout this study. Neonatal daphnids were obtained from

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continuous cultures in 1 L glass beakers at 20±1 °C with ASTM hard water medium (ASTM, 1998) enriched with the organic additive Marinure '25' (Pann Britannica Industries Ltd., Waltham Abbey, UK), an extract from the algae *Ascophyllum nodosum* (Baird et al., 1989) at a concentration of 4.0 ml $L^{-1}$. ASTM hard water has a total hardness of 160–180 mg $L^{-1}$ $CaCO_3$, a pH range of 7.5–8.0 and a conductivity of 580 μS $cm^{-1}$.

The culture medium was renewed three times weekly. Daphnids were fed daily with the green algae *Pseudokirchneriella subcapitata*, formely *Selenastrum capricornutum* ($3 \times 10^5$ cells/mL/*Daphnia*), maintained in light: dark cycle of 16:8 h with a density of below 20 individuals per beaker.

All experiments were initiated with third to fifth brood neonates (⩽24 h old) from a single clone derived from a healthy parent stock. Test conditions were similar to culture conditions. During the experiments temperature, dissolved oxygen, pH, total hardness, and conductivity were monitored weekly.

### 2.2. Chemicals and test solutions

High purity (98–99.9%) standard of testosterone was purchased from Sigma-Aldrich (St. Louis, MO, USA). The compound 4-hydroxy-androst-4-ene-3,17-dione or 4-OHA was synthesized and purified by Tavares da Silva et al. (1996) in the Laboratory of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Coimbra, Portugal.

Stock standard solutions of testosterone and 4-OHA were prepared in dimethyl sulfoxide (Barbosa et al., 2003); the concentration of the solvent in the medium was less than 0.01%, including the control groups. Two stocks solutions of testosterone (0.35 and 0.1 M) and 4-OHA (0.27 and 0.18 M) were made. Test solutions were prepared immediately prior to the tests by diluting the stock solution in ASTM medium for each test. The solvent controls were included in all tests.

### 2.3. Acute toxicity

The acute test was carried out in accordance with OECD guidelines (OECD, 2000) for the determination of the inhibition of mobility of *D. magna*. In brief, this involves the separation of gravid females into 400 mL beakers containing 300 mL of fresh culture medium and the subsequent collection of newly released neonates (<24 h old). The neonatal daphnids were exposed to the control medium, control solvent, testosterone and 4-OHA nominal concentration range of 2.5–6.2 and 2.5–7.4 mg $L^{-1}$, respectively. Each treatment concentration of four 100 mL beakers contained 50 mL of test chemical and five neonatal *Daphnia*. Test organisms were not fed during the course of the experiment. Test vessels were maintained for 48 h at 20±1 °C in a 16:8 h photoperiod. The endpoint examined was immobilization, defined as inability to swim (actively move) after 15 s of gentle agitation.

### 2.4. Chronic toxicity

The chronic test was carried out in accordance with OECD guidelines (OECD, 1998) for long-term toxicity testing. Ten replicated beakers (culture vessels) were filled with the appropriate volumes of ASTM and stock solutions. Daphnids used in the 21-day chronic assays, both those control and those for each compound concentration tested, were individually cultured in 50-mL glass vessels. The assay was performed with nominal testosterone and 4-OHA concentration range of 0.15–2.48 and 0.22–0.84 mg $L^{-1}$, respectively. Each dilution of the test chemical consisted of ten 100 mL beakers containing 50 mL of test chemical and one neonatal *Daphnia*. The test was carried out on a semi-static basis and test solutions were renewed three times a week. The test duration was 21 days and the organisms were fed as previously described. During the experiments the temperature, dissolved oxygen and pH were measured weekly to verify if they were not affecting biological responses. Reproduction was assessed by recording the following parameters during the test: fecundity (total number of eggs-viable and no-viable eggs) produced per female, fertility (total number of viable eggs) produced per female and the number of broods produced by female. Embryonic development abnormalities were assessed by the examination of neonates with a microscope. Thus, the number of aborted eggs and the production of male offspring were recorded. The survival and moulting frequency were parameters examined. The moulting frequency was measured by visual detection of exuvia in the individual test vessels.

Growth was assessed by measuring the length of the first exopodite of the second antenna (AL) of the first moult and of the last moult released within the 21-day period of the tests. The body length (BL) of *D. magna* was obtained using a relationship obtained by measuring the AL and BL of 416 *D. magna* individuals during their life cycle: BL (*D. magna*) = 10.558 × AL (*D. magna*)−0.3457 (mm) ($r^2 = 0.962$) following the procedure of Soares (1989). The survival and moulting frequency were parameters examined. The entire reproduction tests fulfilled the validity clauses of a production of a mean number of offspring in the controls ⩾60, a coefficient of variation less than 25%, and control mortality lower than 10%.

### 2.5. Statistical evaluation of data

The number of immobilized organisms from acute immobilization test was plotted against the test concentrations, and a 48 h $EC_{50}$ with a 95% confidence limit was calculated using standard probit procedure (Finney, 1971).

Data from chronic tests were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test to compare treatments with the controls (Zar, 1996). A nested ANOVA was used for analysis of the data of fecundity and fertility, after logarithmic transformations to correct for deviations from normality. This analysis was performed using MINITAB Statistical Software™ Inc., PA, US, 2000. Significance testing was performed on all data and when applicable significant differences ($p<0.05$) are indicated. Tukey's test was used to assess the differences between treatments. The significance level used for all statistical tests was 0.05.

## 3. Results

### 3.1. Acute toxicity

The highest testosterone concentration tested (6.20 mg $L^{-1}$) corresponds to the maximum solubility of the compound in the ASTM medium. At this concentration, testosterone had no effect on mortality and immobilization of daphnids during the 48 h exposure period.

The acute toxicity test for 4-OHA shows a 48 h $LC_{50}$ value of 4.26±2.94–6.16 mg $L^{-1}$ (95% C.I.). In both cases, controls survival was 100%.

### 3.2. Chronic toxicity

The maternal survival of daphnids exposed to testosterone and 4-OHA was always 100% and no effect was observed in the moults, brood production and BL for both compounds. The exposure of daphnids to testosterone and 4-OHA did not produce male offspring or other developmental abnormalities.

Fig. 1a shows the effects of testosterone upon the fecundity and fertility of *D. magna* at the first brood. A reduction in reproductive output of females exposed to the concentrations 0.62, 1.24, and 2.48 mg $L^{-1}$ was observed. Statistically significant differences were found between controls and treatment groups for age at release of the first brood ($F_{6,63} = 3.65$, $p<0.001$) and fertility ($F_{6,63} = 31.31$, $p<0.001$).

The marked decrease in the number of neonates at 2.48 mg $L^{-1}$of testosterone reflects the increasing number of aborted eggs (95% of the eggs produced were aborted) although no changes in egg morphology were observed.

Fig. 1b shows the effects of testosterone on the number of neonates produced (21 d) after bioassay. The fecundity ($F_{6,63} = 16.84$, $p<0.001$) and the fertility ($F_{6,63} = 33.65$, $p<0.001$), were both affected by testosterone but the calculated NOEC (0.15 mg $L^{-1}$) and LOEC (0.31 mg $L^{-1}$) values are the same for fecundity and fertility. No statistically significant differences were found between the control treatments (ASTM and ASTM+DMSO), for both fecundity (two-way ANOVA: $F_{1,72} = 0.54$; $p = 0.463$) and fertility (two-way ANOVA: $F_{1,72} = 0.44$; $p = 0.510$) at 21 days of assay, thus results from these treatments were grouped together in the ANOVA analysis.

A nested ANOVA using concentration as fixed factor and the brood as a hierarchical factor were used to evaluate these results. The fecundity was significantly affected between concentrations ($F_{6,252} = 9.02$, $p<0.001$) and broods ($F_{21,252} = 70.83$, $p<0.001$). All broods (except the second) showed reduction in fecundity at testosterone concentration of 2.48 mg $L^{-1}$ relative to the control following Dunnet test (Fig. 2a).

The data analysis for fertility assessed with a nested ANOVA, using fecundity as covariable, to evaluate fecundity-independent effects on fertility, showed significant differences between

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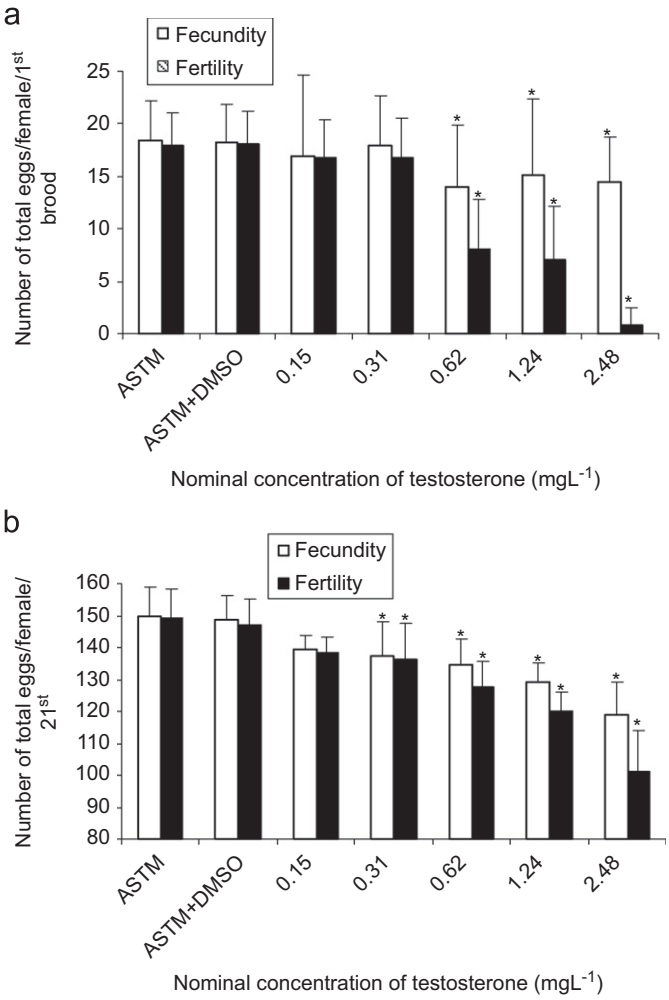


**Fig. 1.** (a) Number of total eggs produced in the first brood by *Daphnia magna* at different concentrations of testosterone (mean ± SD, $n = 10$). (b) Number of total eggs produced by *Daphnia magna* at the end of the bioassay for different concentrations of testosterone (mean ± SD, $n = 10$). The asterisks indicate a value significantly different from the control at $p < 0.05$.

concentrations ($F_{6,242} = 28.42$, $p < 0.001$) and broods ($F_{21,242} = 17.69$, $p < 0.001$) (Fig. 2b). The relationship between fecundity and fertility was also significant ($F_{1,242} = 223.70$, $p < 0.001$).

Statistically significant differences in the number of neonates produced between controls and treatments at the highest concentration of testosterone tested (2.48 mg $L^{-1}$) for first clutch ($F_{6,63} = 31.31$, $p < 0.001$), third clutch ($F_{6,63} = 26.54$, $p < 0.001$) and fourth clutch ($F_{6,63} = 33.65$, $p < 0.001$) as shown in Fig. 2b. However, the BL was not significantly affected by testosterone at either the first brood ($F_{6,63} = 1.95$, $p = 0.09$) or at the end of the assay ($F_{6,63} = 1.47$, $p = 0.20$).

The 4-OHA has no statistically significant effect in the total number of neonates produced by daphnids but a statistically significant effect in the total number of viable eggs at 0.84 mg $L^{-1}$ (Fig. 3a). No statistically significant differences between controls and corresponding treatment groups were recorded in the first brood ($F_{6,63} = 1.95$, $p = 0.09$), at first brood and at the end of the bioassay (21st day) ($F_{6,63} = 1.38$, $p = 0.24$).

The calculated NOEC (0.62 mg $L^{-1}$) and LOEC (0.84 mg $L^{-1}$) values were identical for fecundity and fertility. The marked decrease in the number of viable neonates at 0.84 mg $L^{-1}$ of 4-OHA was due to the increasing number of dead neonates in the fourth brood. Fecundity was not significantly affected between concentrations ($F_{6,252} = 1.86$, $p = 0.09$) as assessed by nested ANOVA. However, significant differences were produced by 4-OHA between broods ($F_{21,252} = 70.83$, $p < 0.001$) (Fig. 3b).

Concerning the fertility, using the fecundity as covariable in the nested ANOVA, 4-OHA exposure resulted in statistically significant differences between concentrations ($F_{6,251} = 8.29$, $p < 0.001$) and between brood ($F_{21,251} = 10.95$, $p < 0.001$) for 0.84 mg $L^{-1}$ and fourth brood, as shown in Fig. 3c). The 4-OHA did not show statistically significant effects on growth of *D. magna* during the period of assay: first brood ($F_{6,63} = 2.03$, $p = 0.07$); 21st day ($F_{6,63} = 1.46$, $p = 0.20$).

## 4. Discussion

The purpose of this study was to test the acute and chronic effects of the steroid compounds, testosterone and 4-OHA, on *D. magna* life-history parameters. There have been evidences for a role of the vertebrate-type sex steroids (androgens, estrogens, progestogens) in the regulation of various reproductive processes in crustaceans (LeBlanc, 2000). Testosterone is excreted into the environment from the same sources and in comparable amounts as 17$\beta$-estradiol (Lintelmann et al., 2003, Shore and Shemesh, 2003; Shore et al., 1995), but the adverse effects of this steroid in the environment is scarce in literature.

Our results suggest that testosterone and 4-OHA induced acute and chronics effects in non-target species at concentrations at least one order of magnitude higher than those found at environment, e.g. a testosterone concentration of 0.116 μg $L^{-1}$ in surface waters has been reported (Kolpin et al., 2002). Furthermore, 4-OHA was more toxic for *D. magna* than testosterone: $EC_{50}$ being the 4.26 mg $L^{-1}$ for 4-OHA and no toxicity was found for higher concentration of testosterone tested (6.20 mg $L^{-1}$). Baldwin and LeBlanc (1994a, b) have shown that *D. magna* has the capacity to metabolize testosterone to polar and apolar metabolites. At sub-lethal concentrations, testosterone reduced significantly the number of viable offspring, and this was more pronounced at 2.48 mg $L^{-1}$. The number of aborted eggs, mainly released in the first brood, also increased with increasing concentrations. The nonviable offspring were mainly represented by aborted eggs, although *D. magna* produced abnormal neonates at the highest concentrations. A positive association between the number of aborted eggs and the testosterone dose was observed suggesting that this compound reduced the reproduction of daphnids by interfering with the normal eggs development. Several authors (Baird et al., 1991; Guilhermino et al., 1999) verified that sodium bromide and 3,4-dichloroaniline (DCA) caused greater abnormal embryonic development, an increased level of abortion and reduced the total number of viable offspring released. In addition, the egg production was unaffected by chronic exposure to both chemical, which conforms to *D. magna* behavior (Baird et al., 1991). They hypothesized that the effect of DCA on reproduction resulted from the direct poisoning of developing embryos in the brood chamber, not in the ovary. Future experiments should examine the development of parthenogenetic eggs in order to define testosterone action.

Kashian and Dodson (2004) also reported that short-term testosterone exposure at 100 μg $L^{-1}$ significantly reduced fecundity while long-term exposure did not produced the same effect, potentially indicating increased testosterone hydroxylation with long-term exposure. *Daphnia* are capable of hydroxylation testosterone at multiple sites by P450 enzymes (Baldwin and LeBlanc, 1994a), giving an explanation for the lack effect in fecundity and fertility observed in our study at the concentrations 0.15 and 0.31 mg $L^{-1}$. It was also hypothesized that chronic

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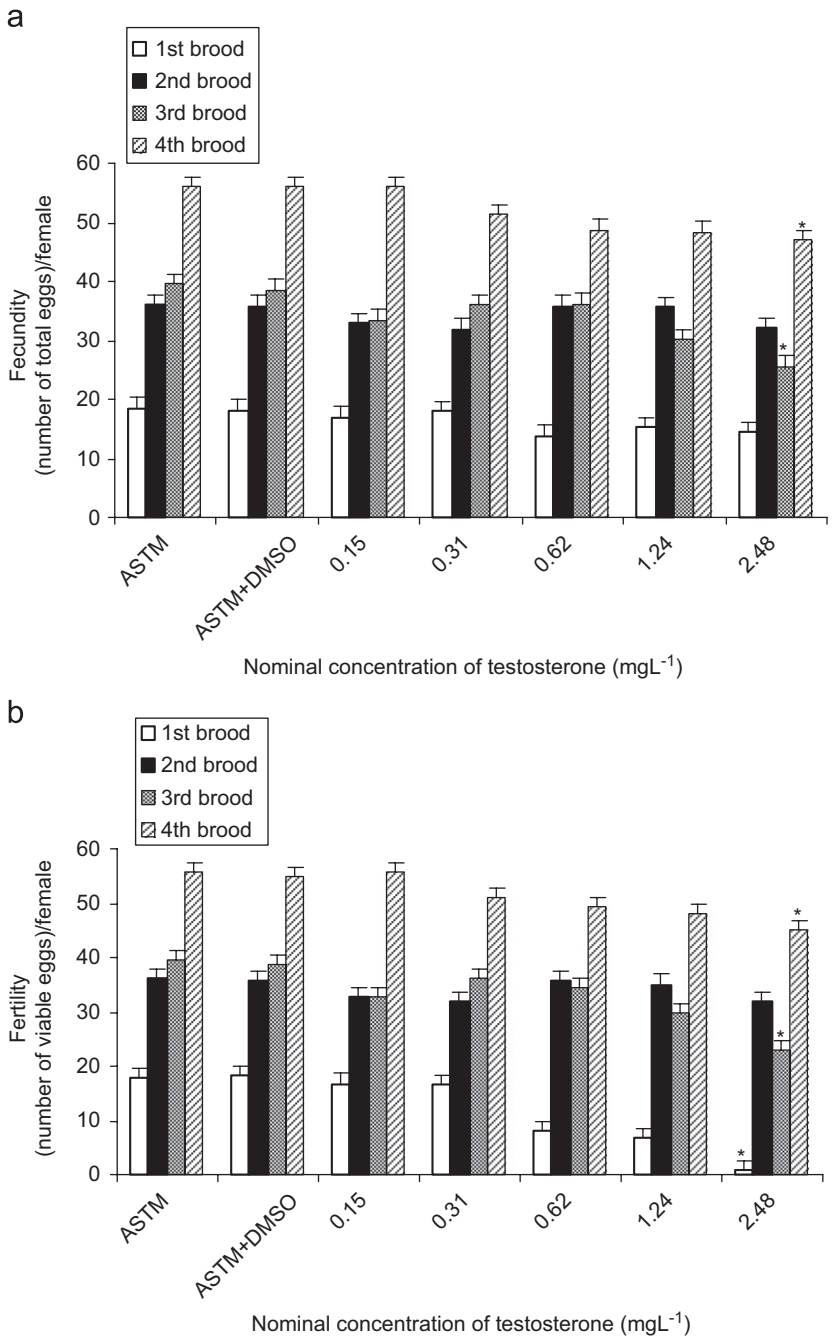


**Fig. 2.** (a) Testosterone effects on fecundity (number of total eggs—viable and no-viable eggs) of *D. magna* for different concentrations and broods (mean ± SD, $n = 10$). (b) Testosterone effects on fertility (total number of viable eggs) of *D. magna* for different concentrations and broods (mean ± SD, $n = 10$). The asterisks indicate a value significantly different from the control at $p < 0.05$.

exposure may require adequate time for cytochrome P-450 (CYP) induction and upregulation prior to release (Kashian and Dodson, 2004). In another study, Kashian (2004) indicated CYP associated acclimation in *Daphnia* occurring 7–12 days (the time period in which the second–third broods are released) following the initial exposure to estrogenic pesticide toxaphene. This acclimation pattern corresponded, perhaps, with the second broods that did not show reduction of fecundity at testosterone concentration of 2.48 mg $L^{-1}$ relative to the control. Because the toxicity can vary with duration of toxicant exposure, concentrations and stage of development of daphnids, the comparison of our results of acute and chronic toxicity tests with those previously reported is difficult.

Some studies indicate that testosterone, which is structurally similar to estrogen, elicits embryo toxicity to daphnids by interfering with ecdysteroid activity (Mu and LeBlanc, 2002). The authors have demonstrated that exposure *D. magna* to 1.15 and 2.30 mg $L^{-1}$ testosterone significantly reduced the fecundity, had no effect on parental survival but delayed moulting and significantly increased developmental abnormalities in offspring. Developmental abnormalities induced by anti-ecdysteroids, including testosterone, can be associated with suppressed ecdysone levels in embryos and these abnormalities (carapace, antennae to severely developmentally arrested individuals) can be protect by co-exposure to 20-hydroxyecdysone (Mu and LeBlanc, 2002). However, in our study no morphological abnormalities among the

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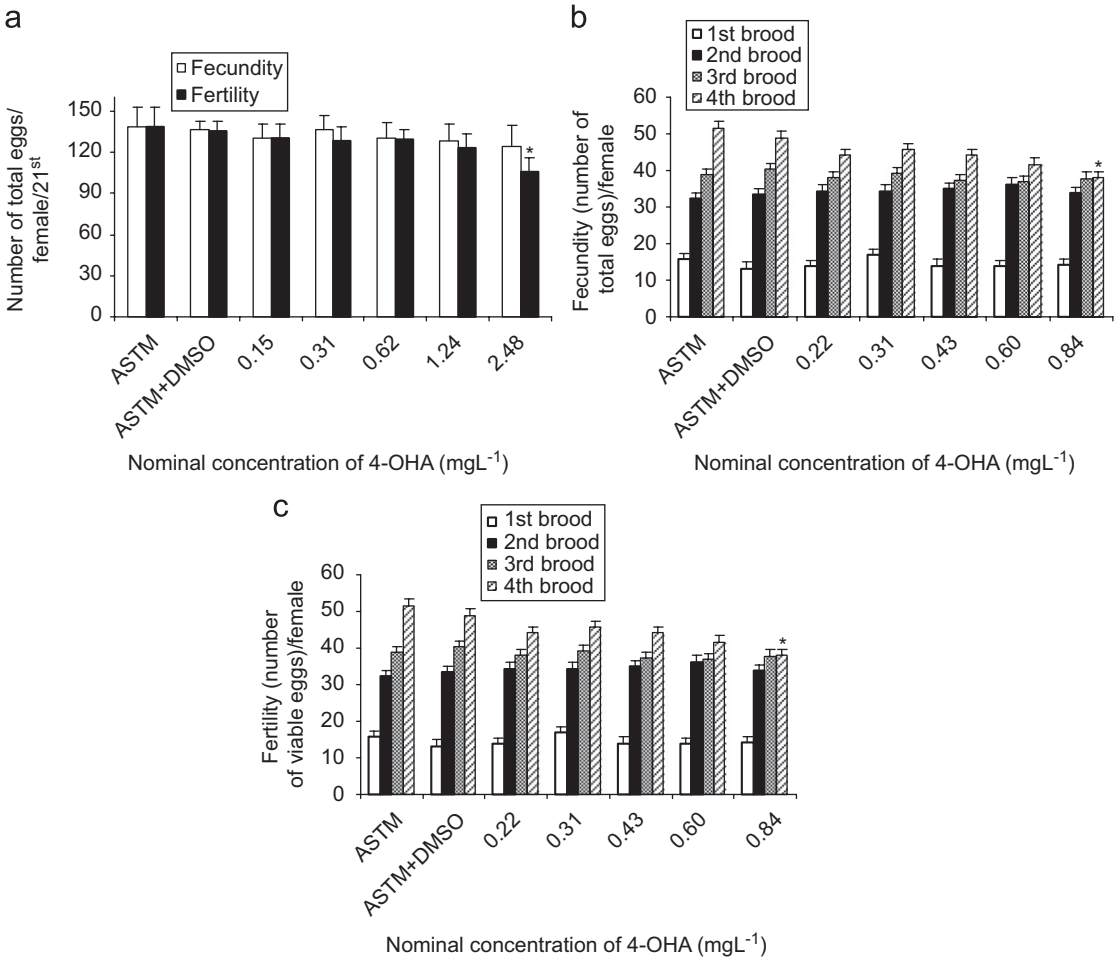


**Fig. 3.** (a) Number of total eggs produced by *Daphnia magna* at the end of 21 day assay for 4-OHA (mean ± SD, $n = 10$). (b) The 4-OHA effects on fecundity number of total eggs—viable and no-viable eggs) of *Daphnia magna* and broods (mean ± SD, $n = 10$). (c) The 4-OHA effects on fertility (total number of viable eggs) of *D. magna* for different concentrations and broods (mean ± SD, $n = 10$). The asterisks indicate a value significantly different from the control at $p < 0.05$.

offspring or delays in moulting were observed. Instead, we observed a decrease in fecundity associated with an increase in aborted eggs. This clearly indicates a disruption of eggs development of the cladoceran *D. magna* as a result of continued exposure to androgenic compounds. Thus, testosterone can be a developmental toxicant in daphnids, acting as an anti-ecdysteroid because aborted eggs can be considered developmental abnormalities. The mechanism underlying the effects observed at 0.62–2.48 mg $L^{-1}$ testosterone is unknown and needs further research.

Androstenedione is another steroidal compound, which is a precursor of testosterone that interferes with the development of daphnids (Olmstead and LeBlanc, 2000). This compound elicits embryos abnormalities at 2.29 mg $L^{-1}$ and appears less potent as a development toxicant when compared with testosterone. These observations suggest that steroidal androgens have a specific toxicity target in *D. magna*.

Another endpoint evaluated in the chronic assays was the effect of testosterone on male production. In vertebrates, this steroid compound is the principal male sex hormone and is responsible for male secondary sex characteristics. However, we did not observe the production of male offspring in response to testosterone exposure which agrees with LeBlanc and collaborators (2000). Furthermore, the eggs aborted and the development abnormalities produced by testosterone are not indicative of masculinization, being more consistent with a developmental disruption. As shown here testosterone interferes with endocrine function in daphnids resulting in development of toxicity in embryos, but this effect is not related to masculinization.

Beyond clinical pharmacokinetics, little is currently known about how the drugs anti-oestrogenics interacts with and changes within non-target organisms (Clubbs and Brooks, 2007).

For 4-OHA, an anti-estrogen, this study demonstrates that this compound did not affect the number of neonates produced by *D. magna*. However, after exposing these organisms to 0.84 mg $L^{-1}$ a significant mortality of neonates was observed at the 4th brood. The toxicity effect reported for testosterone was not observed for 4-OHA, which seems to indicate that this steroid compound elicits its toxicity by interfering with a different stage of normal development of eggs, inducing abnormalities in neonates that were not visible.

Currently available information in the literature on the specific effects of ant-estrogens on invertebrates is scarce. Faslodex or Fluvestrant is a steroidal anti-estrogen model and a selective oestrogen receptor modulator (SERM), also used in advanced human breast cancer treatment (Clarke et al., 2001). Roepke et al. (2005) reported the inhibition of development of sea urchin embryos at concentrations as low 0.03 ng $L^{-1}$ of Faslodex. Exposing *D. magna* for 6 d at Faslodex concentrations ranging from 1 to 100 μg $L^{-1}$ had no significant effects on survivorship,

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fecundity, ephippium production, adult size, changes in morphology, and sex determination of neonates produced during the short-term study (Kashian and Dodson, 2004).

Testosterone and 4-OHA induce toxicity in the first and fourth broods, respectively. This can be explained considering that testosterone is more lipophilic, with a partition coefficient ($\log K_{ow} = 3.2$) higher than 4-OHA (Table 1). Based on this structural descriptor, testosterone can rapidly and freely diffuse across lipophilic biological structures (cell membranes, carapaces) to enter target cells (Oren et al., 2004). The 4-OHA molecule is less lipophilic ($\log K_{ow} = 2.7$) making it more difficult to penetrate the carapace of the mother and affect eggs in the ovary after bioaccumulation. Exposure to these steroids compounds, as a result of their continued discharge to aquatic environment, may result in bioaccumulation. At present, few studies have investigated the bioaccumulation of steroid compounds in daphnids. The available data that reported on the accumulation of steroid estrogens in *D. magna* were for estrone (Gomes et al., 2004). They demonstrated that the uptake via the trophic route is likely to be less significant compared to bioconcentration from aqueous medium.

Several steroid hormones have been detected in water and sediment samples (Lai et al., 2000; Thorpe et al., 2003). Agricultural and other anthropogenic activities are principal non-point sources for steroid hormones in water and sediment systems (Kolpin et al., 2002). With the increasing significance of EDs on environmental health (Desbrow et al., 1998; Wu et al., 2003) the concern about the potential negative ecological effects of steroid hormones has resulted in an increased interest regarding the occurrence, distribution, mobility and persistence of these compounds in soils, sediments and water systems (Ying et al., 2002).

Steroid hormones undergo sorption and transformation in soils (Jenkins et al., 2003; Lee et al., 2003) and sediments (Lai et al., 2000). This fact underlines the importance of these processes in the fate and transport of hormones in environment.

Limited studies have been reported on the fate, transport, degradation and mobility of androgenic hormones in soils. Testosterone binds strongly to the organic phase of soil particles and seems more mobile in soils (Lee et al., 2003; Finlay-Moore et al., 2000; Casey et al., 2003, 2004; Stumpe and Marschner, 2007) and is less resistant against microbial degradation (Stumpe and Marschner, 2007; Horinouchi et al., 2001); the degradation pathway and persistence in natural soils are poorly understood (Fan et al., 2007; Stumpe and Marschner, 2007). Although it was found that testosterone degraded rapidly, it appeared to have a greater potential to migrate to depths in the soil where biodegradation rates are reduced (Finlay-Moore et al., 2000) than the potential for subsurface water contamination to increase.

On the other hand, it is widely recognized that sorption process of hydrophobic organic compounds onto dissolved natural organic matter (DOM) may significantly increase their aqueous solubility (Chiou et al., 1987; Yamamoto et al., 2003). This solubility enhancement affects the fate of these chemical species in an aquatic environment and water treatment processes (Yamamoto et al., 2003) but the increases in dissolved organic matter concentration result in decreases in bioavailability and the bioaccumulation of these chemicals while attenuating their toxicity. The steroid hormones are one class of hydrophobic organic compounds, poorly soluble in aqueous media, whose sorption is of critical importance for understanding the fate of these compounds in the environment. On other hand, despite the levels of testosterone detected in ng $L^{-1}$ range we can hypothesize that the presence in lower concentrations of these steroidal compounds may promote unpredictably chronic effects.

Essentially, the actions of androgens are determined by their physicochemical properties and site-specific environmental conditions; however, their effects on wildlife and human health still remain unclear (Fan et al., 2007).

In the present study, the structural similarity of testosterone, androstenedione, and 4-OHA, as illustrated in Table 1, produced variable acute and reproductively toxicity to the *D. magna*. Thus, we hypothesize that testosterone interferes directly with early embryonic development, while 4-OHA interferes, by bioaccumulation, with the later stages of daphnid embryonic development via maternal exposure.

Pharmaceutical active compounds such as reproductive and steroidal hormones introduced continually via sewage effluent into the aquatic environments make these compounds "pseudo-persistent" pollutants with implications for aquatic organisms (Daughton, 2002). Due to their importance in human use and to the endocrine-disruption effects, the aquatic toxicity of these compounds needs to be further investigated.

## 5. Conclusion

In conclusion, testosterone did not induce acute toxicity in *D. magna* below its solubility limit. In contrast, chronic exposures markedly impaired reproduction in *D. magna* resulting in the abortion of eggs mainly at high concentration and in the first brood. The 4-OHA affects the reproduction of daphnids and increased neonate mortality at highest concentration. Neither compound produced changes in growth, moulting, and frequency of reproduction of *D. magna*. Testosterone interfered directly with normal eggs development with a significant abortive effect, while 4-OHA interfered with normal eggs development with increasing mortality only in the fourth brood. Testosterone and 4-OHA were toxic to aquatic organisms, like *Daphnia*, at concentrations below its water solubility and the possible environmental hazards of these compounds to aquatic biota should be considered.

## References

American Society of Testing Materials, 1998. Standard practice for conducting toxicity tests with fishes, macroinvertebrates and amphibians. E729-90. In: Annual Book of ASTM Standards, Philadelphia, pp. 271–296.

Baird, D.J., Barber, I., Bradley, M., Calow, P., Soares, A.M.V.M., 1989. The *Daphnia* bioassay: a critique. Hydrobiologia 188/189, 403–406.

Baird, D.J., Barber, I., Soares, A.M.V.M., Calow, P., 1991. An early life-stage test with *Daphnia magna* Straus: an alternative to the 21-day chronic test? Ecotoxicol. Environ. Saf. 22, 1–7.

Baldwin, W.S., LeBlanc, G.A., 1994a. Identification of multiple steroid hydroxylases in *Daphnia magna* and their modulation by xenobiotics. Environ. Toxicol. Chem. 13, 1013–1021.

Baldwin, W.S., LeBlanc, G.A., 1994b. In vivo biotransformation of testosterone by phase I and detoxication enzymes and their modulation by 20-hydoxyecdysone in *Daphnia magna*. Aquat. Toxicol. 29, 103–117.

Barbosa, I.R., Martins, R.M., Sá e Melo, M.L., Soares, A.M.V.M., 2003. Acute and chronic toxicity of dimethylsulfoxide to *Daphnia magna*. Bull. Environ. Contam. Toxicol. 70 (6), 1264–1268.

Blankvoort, B.M.G., Rodenburg, R.J.T., Murk, A.T.J., Koeman, J.H., Schilt, R., Aarts, M.M.J.G., 2005. Androgenic activity in surface water samples detected using the AR-LUX assay: indications for mixture effects. Environ. Toxicol. Pharmacol. 19, 263–272.

Brodie, A.M., Schwarzel, W.C., Shaikh, A.A., Brodie, H.J., 1977. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent process in reproduction and breast cancer. Endocrinology 100, 1684–1695.

Casey, F.X.M., Larsen, G.L., Hakk, H., Šimůnek, J., 2003. Fate and transport of 17$\beta$-estradiol in soils-water systems. Environ. Sci. Technol. 37, 2400–2409.

Casey, F.X.M., Hakk, H., Šimůnek, J., Larsen, G.L., 2004. Fate and transport of testosterone in agriculture soils. Environ. Sci. Technol. 38, 790–798.

Chiou, C.T., Kile, D.E., Brinton, T.I., Malcolm, R.L., Leenheer, J.A., 1987. A comparison of water solubility enhancements of organic solutes by aquatic humic materials and commercial humic acids. Environ. Sci. Technol. 21, 1231–1234.

Clarke, R., Leonessa, F., Welch, J.N., Skarr, T.C., 2001. Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacol. Rev. 53, 25–71.

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Clubbs, R.L., Brooks, B.W., 2007. *Daphnia magna* responses to vertebrate estrogen receptor agonist and antagonist: a multigenerational study. Ecotoxicol. Environ. Saf. 67, 385–398.

Colborn, T., 1995. Environmental estrogens: health implications for humans and wildlife. Environ. Health Perspect. 103 (Suppl.7), 135–136.

Cowan, D.A., Kicman, A.T., 1997. Doping in sport: misuse, analytical tests and legal aspects. Clin. Chem. 43, 1110–1113.

Daughton, C.G., 2002. Environmental stewardship and drugs as pollutants. Lancet 360, 1035–1036.

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P., Waldock, M., 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ. Sci. Technol. 32, 1549–1558.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic physiological and environment influences. Aquaculture 20, 191–364.

Falconer, I.R., Chapman, H.F., Moore, M.R., Ranmuthugala, G., 2006. Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. Environ. Toxicol. 21 (2), 181–191.

Fan, Z., Casey, F.X.M., Hakk, H., Larsen, G.L., 2007. Persistence and fate of 17$\beta$-estradiol and testosterone in agriculture soils. Chemosphere 67, 886–895.

Finlay-Moore, O., Hartel, P.G., Cabrera, M.L., 2000. 17$\beta$-estradiol and testosterone in soil and runoff from grasslands amended with water bodies receiving cattle feedlot effluent in eastern Nebraska, broiler litter. J. Environ. Qual. 29, 1604–1611.

Finney, D.J., 1971. Probit Analysis, third ed. Cambridge University Press, Cambridge, UK.

Folmar, L.C., Hemmer, M.J., Denslow, N.D., Kroll, K., Chn, J., Cheek, A., Richman, H., Meredith, H., Grau, E.G., 2002. A comparasion of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro. Aquat. Toxicol. 60, 101–110.

Gomes, R.L., Deacon, H.E., Lai, KaM., Birkett, J.W., Scrimshaw, M.D., Lester, J.N., 2004. An assessment of bioaccumulation of estrone in *Daphnia magna*. Environ. Toxicol. Chem. 23, 105–108.

Guilhermino, L., Sobral, O., Chastinet, C., Ribeiro, R., Gonçalves, F., Silva, M.C., Soares, A.M.V.M., 1999. A *Daphnia magna* first-brood chronic test: an alternative to the convencional 21-day chronic bioassay? Ecotoxicol. Environ. Saf. 42, 67–74.

Guillette, L.J., Craine, D.A., 2000. Environmental Endocrine Disruptors: An Evolutionary Perspective. Tayler & Francis, New York.

Hanselman, T.A., Graetz, D.A., Wilkie, A.C., 2003. Manure-borne estrogens as potential environmental contaminants: a review. Environ. Sci. Technol. 37, 5471–5478.

Henschel, K.P., Wenzel, A., Diedrich, M., Fliedner, A., 1997. Environmental hazard assessment of pharmaceuticals. Regul. Toxicol. Pharmacol. 25, 220–225.

Horinouchi, M., Yamamoto, T., Taguchi, K., Arai, H., Kudo, T., 2001. Meta-cleavage enzyme gene test *bis* necessary for testosterone degradation in *Comamonas testosterone* TA441. Microbiology 147, 3367–3375.

Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., 2003. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicol. Sci. 73, 53–59.

Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., Moore, R., Nance, M., Brown, A., 2004. Production of androgens by microbial transformation of progesterone in vitro: a model for androgen production in rivers receiving paper and mill effluent. Environ. Health Perspect. 112, 1508–1511.

Kashian, D.R., 2004. Toxaphene detoxification and acclimation in *Daphnia magna*: do cythocrome P-450 enzymes play a role? Comp. Biochem. Physiol. C 137, 53–63.

Kashian, R.D., Dodson, S.I., 2004. Effects of vertebrate hormones on development and sex determination in *Daphnia magna*. Environ. Toxicol. Chem. 23, 1282–1288.

Kolodziej, E.P., Harter, T., Sedlak, D.L., 2004. Dairy wasterwater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. Environ. Sci. Technol. 38, 6377–6384.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Lai, K.M., Johnson, K.L., Scrimshaw, M.D., Lester, J.N., 2000. Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. Environ. Sci. Technol. 34, 3890–3894.

LeBlanc, G.A., 2000. Steroid hormone-regulated process in invertebrates and their susceptibility to environmental endocrine disruption. In: Guillette, L.J., Crain, D.A. (Eds.), Environmental Endocrine Disrupters: An Evolutionary Perspective. Taylor & Francis, London, pp. 126–154.

LeBlanc, G.A., Mu, X., Rider, C.V., 2000. Embryotoxicity of the alkylphenol degradation product 4-nonylphenol to the crustacean *Daphnia magna*. Environ. Health Perspect. 108, 1133–1138.

Lee, L.S., Strock, T.J., Sarmah, A.J., Rao, P.S.C., 2003. Sorption and dissipation of testosterone, estrogens, and their primary transformation products in soils and sediment. Environ. Sci. Technol. 37, 4098–4105.

Lee, P.S., Pankhurst, N.W., King, H.R., 2006. Effects of aromatase inhibitors on in vitro steroidogenesis by Atlantic Salmon (*Salmo salar*) gonadal and brain tissue. Comp. Biochem. Physiol. A 145, 195–203.

Lintelmann, L., Katayama, A., Kurihara, N., Shore, L., Wenzel, A., 2003. Endocrine disruptors in the environment (IUPAC Technical Report). Pure Appl. Chem. 75, 631–681.

Lonning, P.E., 1998. Pharmacological profiles of exemestane and formestane, steroidal aromatase inhibitors used for treatment of postmenopausal breast cancer. Breast Cancer Res. Treat. 49 (Suppl. 1), S45–S52.

Mu, X., LeBlanc, G.A., 2002. Developmental toxicity of testosterone in the crustacean *Daphnia magna* involves anti-ecdysteroidal activity. Gen. Comp. Endocrinol. 129, 127–133.

OECD, 1998. *Daphnia magna* reproduction test. OECD guidelines for testing of chemicals, 211. OECD, Paris.

OECD, 2000. *Daphnia* sp., acute immobilization test. OECD guidelines for testing of chemicals, 202. OECD, Paris.

Olmstead, A.W., LeBlanc, G.A., 2000. Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia Magna*. Environ. Toxicol. Chem. 19, 2107–2113.

Oren, I., Fleishmen, S.J., Kessel, A., Ben-Tal, N., 2004. Free diffusion of steroid hormones across biomembranes: a simplex search with implicit solvent model calculations. Biophys. J. 87, 768–779.

Panter, G.H., Thompson, R.S., Beresford, N., Sumpter, J.P., 1999. Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. Chemosphere 38, 3579–3596.

Petrović, M., Eljarrat, E., Lopez de Alda, M.J., Barceló, D., 2004. Endocrine disrupting compounds and others emerging contaminants in the environment: a survey on new monitoring stategies and ocurrence data. Anal. Bioanal. Chem. 378, 549–562.

Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197, 229–281.

Recanatini, M., Cavalli, A., Valenti, P., 2002. Nonsteroidal aromatase inhibitors: recent advances. Med. Res. Rev. 22, 282–304.

Roepke, T.A., Snyder, M.J., Cherr, G.N., 2005. Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmental relevant concentrations. Aquat. Toxicol. 71, 155–173.

Shore, L.S., Shemesh, M., 2003. Naturally produced steroid hormones and their release into the environment. Pure Appl. Chem. 75 (11, 12), 1859–1871.

Shore, L.S., Correll, D., Chakroborty, P.K., 1995. Fertilization of fields with chicken manure is a source of estrogens in small streams. In: Steele, K. (Ed.), Animal Waste and the Land–Water Interface. Lewis Publishers, Boca Raton, FL, pp. 49–56.

Soares, A.M.V.M. (1989). Clonal variation in life history traits in *Daphnia magna* Straus (Crustacea, Cladocera). Implications for ecotoxicology. PhD. Thesis, Department of Animal and Plant Sciences, University of Sheffield.

Stalschmidtallner, P., Allner, B., Rombke, J., Knacker, T., 1997. Endocrine disruptors in the aquatic environemt. Environ. Sci. Pollut. Res. 4, 155–162.

Stumpe, B., Marschner, B., 2007. Long-term sewage application and wastewater irrigation on the mineralization and sorption of 17β-estradiol and testosterone in soils. Sci. Tot. Environ. 374, 282–291.

Sumpter, J.P., 1998. Xenoendocrine disrupters-environmental impacts. Toxicol. Lett. 102, 103, 337–342.

Sumpter, J.P., 2005. Endocrine disrupters in the aquatic environment: an overview. Acta Hydrochim. Hydrobiol. 33, 9–16.

Svenson, A., Allard, A.-S., 2004. In vitro androgenicity in pulp and paper mill effluents. Environ. Toxicol. 19, 510–517.

Tavares da Silva, E., Sá e Melo, M.L., Campos Neves, A., 1996. Novel approach to the synthesis of the aromatase inhibitor 4-hydroxyandrost-4-ene-3,17-dione (4-OHA). J. Chem. Soc. PerKin Trans. 1, 1649–1650.

Thomas, K.V., Hurst, M.R., Smith, A., McHugh, M., Matthiessen, P., Waldock, M.J., 2002. An assessment of androgenic activity and the identification of environmental androgens in United Kingdom estuaries. Environ. Toxicol. Chem. 21, 1456–1461.

Thorpe, K.L., Cummings, R.L., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., Tyler, C.R., 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 37, 1142–1149.

Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28, 319–361.

Wu, R.S.S., Zhou, B.S., Randall, D.J., Woo, N.Y.S., Lam, P.K.S., 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environ. Sci. Technol. 37, 1137–1141.

Yamamoto, H., Liljestrand, H.M., Shimizu, Y., Morita, M., 2003. Effects of physical-chemical characteristics on the sorption of selected endocrine disruptors by dissolved organic matter surrogates. Environ. Sci. Technol. 37, 2646–2657.

Ying, G.-G., Kookama, R.S., Ru, Y.-J., 2002. Occurrence and fate of hormone steroids in the environment. Environ. Int. 28, 545–551.

Zar, J.H., 1996. Biostatistical Analysis, third ed. Prentice-Hall, Englewood Cliffs, NJ, USA.

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