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MAMMALIAN GLUCOCORTICOID METABOLITES ACT AS ANDROGENIC ENDOCRINE DISRUPTORS IN THE MEDAKA (*ORYZIAS LATIPES*)

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Abstract—Glucocorticoid metabolites enter the aquatic environment via mammalian excrements. Molecular structures of their $C_{19}O_3$ metabolites strongly resemble the major fish androgen 11-ketotestosterone. Therefore, we tested the hypothesis that the cortisol metabolite 5α-androstan-3,11,17-trione acts similarly to 11-ketotestosterone by employing a fish screening assay for endocrine-active substances. After 21 d, both 11-oxygenated compounds had masculinized sex characteristics of the anal fin in female medaka in a dose-dependent manner. Environ. Toxicol. Chem. 2010;29:1613–1620. © 2010 SETAC

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INTRODUCTION

Natural and synthetic steroid hormones and their metabolites originating from mammalian excrements enter the aquatic environment through urban [1] and agricultural [2–4] water cycles at concentrations hormonally active in aquatic wildlife [5–8]. Although information on the fate of steroids in municipal sewage is abundant, it is scarce for livestock waste. Among the steroid hormones released from sewage treatment plants and different livestock production systems into the aquatic environment, sex steroids have been thoroughly described as potential endocrine disruptors [1,2,8], whereas glucocorticoids have rarely been considered, focusing on pharmaceutical use [9] and sewage treatment plants [10–12].

A variety of anthropogenic chemical substances are suspected or known endocrine disruptors, and several modes of action have been established [13–15]. Overall, research on endocrine disruptors has focused on effects on reproductive structures and function in teleost fish. Although estrogenic and antiestrogenic effects have been studied rather frequently, antiandrogenic and particularly androgenic effects have received comparatively little attention [16–19]. Xenoandrogens are considered to be diverse and widespread [20–22]. Those identified for fish include synthetic anabolic androgens such as trenbolone acetate used in livestock production and its metabolite 17β-trenbolone [23,24], 17α-methyltestosterone used in fish farming [25,26], the biocide tributyltin [27–29], and pulp and paper mill effluents [30–32].

Xenohormones represent a major category of emerging micropollutants in aquatic ecosystems [33–35]. Therefore, during the past decade, much effort has been placed into reviewing, developing, and validating an array of screening assays within the Organization for Economic Co-operation and Deveopment (OECD) Endocrine Disruptor Testing and Assessment ([36]; see http://www.oecd.org/document/62/0,2340,en_2649_34377_2348606_1_1_1_1,00.html) and the U.S. Environmental Protection Agency (U.S. EPA) Endocrine Disruptor Screening Program ([37]; see http://www.epa.gov/endo/pubs/assayvalidation/index.htm). For fish, two major lines of action have been pursued, the 21-d fish endocrine screening assay with fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), and medaka (*Oryzias latipes*), followed by the androgenized female stickleback screening assay with stickleback (*Gasterosteus aculeatus*) [36–38]. In this standardized endocrine disruptors testing with fish, methyltestosterone and trenbolone have been most frequently employed as exogenous androgenic model test compounds ([14,23,36,38–40]; see also http://www.oecd.org/document/62/0,2340,en_2649_34377_2348606_1_1_1_1,00.html#Stickleback).

Among the sex steroid hormones of teleost fish, 11-ketotestosterone represents the major androgen [41–44]. Correspondingly, the potential of exogenous 11-ketotestosterone in modulating fish endocrine system has been described for several levels of integration from androgen receptor binding [45], to gene expression [46,47], through gametogenesis [46,48], early life phases development and growth [49], gonadal differentiation [50], internal sex characters (in particular spiggin, the kidney-secreted protein making up the nest building glue in male three-spined stickleback and associated renal structures [18,51] or external accessory sex characters [41,42,52]), to mating behavior, parental care, and pheromonal communication [53,54]. External accessory sex characters are particularly practicable to assess [44]. Among the most well-established small laboratory model fish species, adult medaka show distinct external sexual dimorphism in shape and size of body, anal papilla, and unpaired fins [36,55–57]. For this fish model, however, 11-ketotestosterone has rarely been employed as an androgenic model test compound [36,41,58].

The evolutionarily conservative character of hormonal communication at the cellular and subcellular levels is well recognized [35,59,60]. The role of metabolism in the endocrine

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effects of chemical substances in aquatic system has already been described [61,62]. However, the endocrine activities of steroid hormone metabolites other than those of estradiol and testosterone have rarely been addressed in endocrine disruptors research, with focus placed on phytosterols and pulp and paper mill effluents [9,31,63–66]. It has been argued that metabolism of glucocorticoids might result in the formation of substances with androgenic or other nonoestrogenic endocrine activities [9], but mammalian glucocorticoid metabolites as endocrine disruptors in fish have not been investigated until now.

Natural and synthetic glucocorticoids and their metabolites are released into the aquatic environment via mammalian excrements. The environmental presence of glucocorticoids seems to exceed that of estrogens, as indicated by pharmaceutical use profiles and the concentrations detected in sewage treatment plant effluents, river waters, and livestock excrements. Pharmaceutical use profiles showed that total consumption of glucocorticoids, estradiol, and enthinylestradiol in pharmaceuticals in Denmark was 361, 45, and 0.7 kg/year, respectively [9]. Average concentrations of prednisolone, cortisol, and cortisone detected in sewage treatment plant effluents were 0.56, 0.50, and 0.26 ng/L, respectively (sampling locations in Beijing, China [10]). Mean effluent/surface water ratios of glucocorticogenic and estrogenic activity were 11 to 243/0.39 to 1.3 ng dexamethasone-equivalents/L and 0.4 to 1.0/0.2 to 0.5 ng estradiol-equivalents/L, and glucocorticoid levels detected in surface waters ranged from 0.75 to 2.7/0.41 to 2.4 ng dexamethasone-equivalents/L (using CALUX reporter gene bioassays; sampling locations in The Netherlands [11,67]). Maximum total concentrations in river waters for androgens, progestogens, glucocorticoids, and estrogens were 480, 52, 50, and 9.8 ng/L, respectively (using liquid-chromatography tandem mass-spectrometry, study areas in Beijing, China [12]). In livestock excrements, cortisol metabolite levels as indicated by 11-oxoaetiocholanolone-immunoreactive substances in the feces of unstressed animals ranged from 2.3 to 35.2 nmol/kg in horses (using an enzyme immunoassay specific for C_{19} steroids [68]) and from 39 to 104 nmol/kg (median 88 nmol/kg) in lactating cows [69], whereas median levels in cow feces reached 2.2 μmol/kg after transportation stress and decreased to a more constant level of approximately 0.5 μmol/kg (using a similar assay that detected a broader spectrum of glucocorticoid steroids and their metabolites, including C_{21} and C_{19} steroids [70]).

The contribution of farm animal excrements to the presence of glucocorticoids in surface waters has not been assessed to date, but indirect evidence may be inferred when combining the following sets of information: the combined farm animal population generates about four times more estrogens than the human population in the United Kingdom [72]; the estimated yearly amount of estrogens, androgens, and gestagens excreted by farm animals in the European Union and the United States amounted to 82, 11.5, and 601 tons overall, respectively, for $3{,}299 \times 10^6$ head of cattle, pigs, sheep, and chickens, where the mean excreted amounts per adult cattle were 1.30, 0.39, and 7.60 kg, respectively [2]; the measured concentrations of sex steroid hormones derived from farm animals ranked at the nanograms per liter level in runoff and stream water in several studies [72]. Calculated from the data obtained by Palme et al. [69], elimination of $C_{19}O_3$ cortisol metabolites per individual cattle is 3.52 μmol/d or 1.285 mmol/y (88 nmol/kg feces × 40 kg feces/d × 365 d), which is based on 11-oxoaetiocholanolone-equivalents (11-oxoaetiocholanolone molecular mass = 304) corresponding to approximately 1.1 mg/d or 0.4 g/year. After defecation, further microbial metabolism may substantially increase the fecal $C_{19}O_3$ steroid levels, as has been shown for sheep [71]. Therefore, the amount of natural $C_{19}O_3$ metabolites introduced into the environment via livestock feces likely exceeds the estimates in the present study.

The side chain of steroids with 21 carbon atoms (like the glucocorticoids cortisol $C_{21}H_{30}O_5$ and corticosterone $C_{21}H_{30}O_4$) may be subject to microbial metabolism (by side-chain-cleaving enzymes, e.g., synthesized by mammalian intestinal bacteria) and thereby converted to C_{19} steroids [73]. Among these C_{19} metabolites of corticosteroids, $C_{19}H_xO_3$ metabolites such as 5α-androstan-3,11,17-trione were abundant in the feces of ruminants [70] and strongly resembled the major fish androgen 11-ketotestosterone, even sharing the structural formula ($C_{19}H_{26}O_3$).

In this pilot study, we therefore tested the hypothesis that the cortisol metabolite 5α-androstan-3,11,17-trione acts similarly to 11-ketotestosterone in that it masculinizes sex characteristics of unpaired fins in female fish. For this purpose, we employed the medaka (*Oryzias latipes*) model, largely following the OECD draft fish screening assay for endocrine-active substances [36].

MATERIALS AND METHODS

The present study was conducted at the Laboratory for Ecotoxicology of the Department of Biomedical Sciences, University of Veterinary Medicine of Vienna, Austria in 2008. Experimental design largely followed the OECD draft fish screening assay for endocrine-active substances [36], which was adopted most recently [39]. The fish treated in this study were sexually mature male and spawning female wild-type Japanese medaka (*Oryzias latipes*; 8–10 months old, arithmetic mean ± standard deviation; range of variation in brackets: 35.7 ± 1.9 [31–42] mm total length; 0.43 ± 0.09 [0.24–0.74] g mean wet body mass at the end of exposure), bred from a laboratory stock maintained at the above-mentioned department. Two weeks before the start of exposure, the fish were transferred into the test aquaria so that each test aquarium contained four male and four female fish as indicated by distinct external sexual characteristics. This balanced male/female proportion conforms to the OECD test protocol [36,39] and takes into consideration that monosex designs might be inappropriate to the species and thus harm the fish and bias results. (Yamamoto [74] reports on impaired spawning of females in the absence of males.) Individual fish were randomly placed into the test aquaria, and treatments were randomly assigned to the test aquaria.

The fish had been reared in flow-through tanks under the following general physicochemical test conditions: dilution water was temperature adjusted, dechlorinated, aerated, tap drinking water of the City of Vienna, Austria; photoperiod was 16:8-h light:dark, 800 to 900 lux; no aeration in the tanks and test aquaria. During the experiment, water quality in the aquaria was controlled and maintained as follows (arithmetic mean ± standard deviation; range of variation in brackets): In all aquaria, water temperature (24.8 ± 0.5 [24.1–25.9] degrees centigrade) was measured daily; conductivity (294.2 ± 11.3 [280–320] μS), dissolved oxygen (83.8 ± 8.5 [63.2–92] saturation), and water pH (8.29 ± 0.1 [8.0–8.5]) were measured twice during the exposure period. During the acclimation and exposure periods, fish were fed twice daily (3% of their fresh body mass per day; ground dry fish flake food; Sera Vipan®). After two weeks of acclimation to the general test conditions, fish were continuously exposed to the test compounds via the ambient water for three weeks (20 L test medium per aquarium;

semistatic medium renewal, 100% [i.e., 75% effective] exchange per day; daily cleaning).

Tested compounds and nominal test concentrations were 0.1, 1.0, 10, and 100 μg/L for 5α-androstan-3,11,17-trione (11-OA; Steraloids) and 11-ketotestosterone (11-KT; 4-androsten-17β-ol-3,11-dione; Steraloids). Control groups were exposed to 17β-estradiol at 0.1 μg/L [1,3,5(10)-estratrien-3,17β-diol; Sigma-Aldrich], 17β-trenbolone at 1.0 μg/L (17β-hydroxyestra-4,9,11-trien-3-one; Sigma-Aldrich), ethanol (pro analysi; solvent; Merck) at 20 μg/L, and pure dilution water. Every treatment (i.e., test concentration of a test compound) was run in two simultaneous replicates (i.e., aquaria) with the exception of the highest test concentrations for 11-OA and 11-KT (i.e., 100 μg/L) and the solvent control (Supplemental Data, Table S1).

The present study was designed as a pilot study because of the paucity of information on the effect concentrations in the OECD fish assay for endocrine-active substances for both the target test compound (11-OA) and its primary positive reference test compound (11-KT). Therefore, spacing of the test concentrations of 11-OA and 11-KT was set relatively wide (0.1–100 μg/L), and an additional strong androgen (17β-trenbolone) as well as a strong estrogen (17β-estradiol) were used as internal standards [40]. Also, for 17β-trenbolone, the pertinent database was limited, so this test concentration was set at 1.0 μg/L, considering the existing information on the induction of papillary processes in the anal fin of female medaka after exposure to 17β-trenbolone, following a draft 21-d OECD fish assay protocol (lowest-observed-effective concentration [LOEC] = 45 ng/L, exposure gradient: 4, 14, 45, 150, and 500 ng/L ([75]; see http://abstracts.co.allenpress.com/pweb/setac2005/index.html); LOEC = 365 ng/L actual, exposure gradient: 5,000, 500, and 50 ng/L nominal, no solvent used [24]).

No mortalities were recorded during the acclimation and exposure period, and no signs of disease were visible in the control fish. In two groups (10 μg/L11-OA and the dilution water control), one fish was lost by accident (siphoned off at the beginning of the cleaning procedure). Numbers of fish per replicate and treatment are given in Supplemental Data, Table S1. After two weeks of exposure, five male fish overall showed signs of disease (decreased locomotor and feeding activity) in the exposure groups 100 μg/L 11-OA ($n = 3$), 1.0 μg/L trenbolone ($n = 1$), and ethanol control ($n = 1$) and were killed on that day. A few days later, additional fish of the highest androgen test concentration groups (i.e., 100 μg/L 11-OA, 100 μg/L 11-KT, 1 μg/L trenbolone) seemed to show decreased locomotor activity, and exposure was terminated for all fish in these groups ahead of schedule (by day 18). All other fish reached the end of exposure (day 21) in apparently healthy conditions.

All fish were killed with an overdose of MS222 (100 mg/L neutral buffered tricaine methane sulfonate; Apoka, ACM), measured for body mass to the nearest of 0.01 g (Mettler PM 2500 balance), photodocumented for length measurements to the nearest of 0.1 cm (ImageJ 1.42, http://rsbweb.nih.gov/ij/), fixed in buffered 4% formaldehyde for future histological analysis, and subjected to external sex determination using a stereo microscope (Wild M8).

Adult medaka show distinct external sexual dimorphism in shape and size of body, anal papilla, and unpaired fins [36,40,55–57]. For external sex determination at the end of exposure, in the dorsal and caudal fins of all fish, the following set of male-exclusive structures (i.e., they typically are present only in adult males but not in females and have been described in depth by Egami [55]) was analyzed for their presence or absence (resulting in binary response variables): papillary processes on the anal fin rays, serration of the distal anal fin margin, caudal notch in the anal fin, caudal notch in the dorsal fin. In addition, the following counts were carried out in the anal fin (resulting in continuous, meristic response variables): the total number of fin rays carrying papillary processes and the number of papillary processes on the second-most posterior (caudad) fin ray. For each binary response variable, the scoring scheme distinguished primarily between three character values (female = absent, male = present, and intermediate = uncertain). In cases when one of these fin structures could not be assigned to either the male or the female state with certainty, it was assigned to be intermediate, whether it was a female–male transition stage (which likely exists) or a variation not induced by the treatment (which we cannot exclude). For any of these fin characteristics, the number of individuals showing the male character state was compared with the number of individuals not showing the male character state, with this not-male state comprising both the female and the intermediate states. Considering that the hypothesis tested was targeted at detecting masculinization, the present approach is conservative (i.e., using the female/not-female proportion comparisons between the exposure and negative [dilution water] control groups would result in more cases of statistically significant differences). For presence or absence of the papillary processes, no intermediate states were observed.

The objective of this pilot study was to test the hypothesis that the cortisol metabolite 11-OA acts as an androgen and can masculinize (i.e., stimulate the production of male secondary sex traits in) female medaka fish. Therefore, in the most conservative approach, for each observation, the response could take only the two values: male or not-male. For each character, the male/not-male ratio in the treatment tanks was compared with that in the negative (dilution water) control tanks. Because the response variable was a binary variable (male/not-male), Pearson's chi-square test for independence was used [76]. In instances in which the number of expected cases per cell was too low to fulfill the preconditions for Pearson's chi-square test, we used the Fisher–Yates test instead, which is the suitable test for evaluation of 2×2 tables with small numbers [76]. For the continuous, meristic variables (i.e., number of anal fin rays carrying papillary processes and number of processes present on the second last anal fin ray), comparisons between exposure groups and negative dilution water control groups were performed using the Mann–Whitney rank sum test. All statistical analyses were based on nominal concentrations using SPSS 14.0 for Windows.

RESULTS AND CONCLUSIONS

The mammalian glucocorticoid metabolite 11-OA acts like the major fish androgen 11-KT

At the beginning of exposure, the ratio of externally phenotypic male and female medaka was equal in each test aquarium. After 14 to 21 d of continuous exposure to the test compounds via the holding water, the ratio of male medaka (as indicated by the anal fin characteristics) had risen in both the target test compound groups, exposed to 5∝-androstan-3,11,17-trione (11-OA), the cortisol metabolite and its reference test compound groups exposed to 11-ketotestosterone (11-KT), the major androgen in fish, but not in any of the control groups (estrogen, trenbolone, solvent, pure dilution water). The observed androgenic effects were statistically significant at the higher concentrations tested. Presence of anal fin papillary

processes and number of anal fin rays carrying papillary processes were the most sensitive effect criteria; differences from the control were significant for 1.0, 10, and 100 μg/L 11-OA and for 10 and 100 μg/L 11-KT, and NOEC/ LOEC (no-observed-effect concentration/lowest observed effect concentration) values were 0.1/1.0 μg/L for 11-OA and 1.0/10 μg/L for 11-KT (nominal concentrations; Fig. 1; Supplemental Data, Table S1). Linear regression analysis showed a strong positive association between the meristic response variables on the anal fin and the log-transformed concentrations of exposure (number of fin rays carrying papillary processes: $y = 0.54x \ln(x) + 5.25$, $R^2 = 0.7$, for 11-OA and $y = 0.69x \ln(x) + 4.2$, $R^2 = 0.8$, for 11-KT; number of papillary processes on second-last fin ray: $y = 2{,}28x \ln(x) + 14.5$, $R^2 = 0.9$, for 11-OA and $y = 1.78x \ln(x) + 11.45$, $R^2 = 0.9$, for 11-KT). In summary, both effective test compounds seemed to act in a dose-dependent manner, the exposure–response relationship of 11-OA seemed to parallel that of 11-KT, and the results indicated that the effect potential of 11-OA exceeded that of 11-KT by an order of magnitude.

Although fish readily absorb steroid hormones from the ambient water through their gills [77], studies exposing fish to 11-KT via the aquatic pathway are comparatively rare. For 11-KT, the sensitivity of the medaka fin model employed in this study complied with that reported in the literature. In medaka exposed to 11-KT via the ambient water, incubation of fertilized eggs until hatch resulted in functional phenotypic males (30–300 μg/L) [78]; short-term (96-h) exposure of embryos caused several effects in adults (90 d postexposure; lowest tested concentration at which effects were observed in parentheses): increased growth and germ cell necrosis in males (1,000 μg/L) but reduced body growth in females (100 μg/L) as indicated by body mass and length, and condition factor, thyroid follicular hypertrophy (10 μg/L), germ cell necrosis, and ovarian atresia in females (10 μg/L) [23]; exposure of medaka 7 d posthatch caused up- and down-regulation of several genes in both sexes (100 μg/L) [47]. In female medaka, mean total number of anal fin papillary processes had increased from approximately 10 (for 2 μg/L) to 65 (for 250 μg/L) after 10 d of aquatic exposure to 11-KT (values estimated from graph) [58]. Dietary exposure of adult females to 11-KT over 15 d induced formation of these male characters (LOEC = 25 μg/g, effective dose ED50 = 37 μg/g food [41]). In juvenile guppy, *Poecilia reticulate*, 25 μg/L 11-KT stimulated the appearance of adult male color patterns [79]. Hence, in comparing the available data set, the lowest 11-KT concentrations reported to induce male characters in female fish after aquatic exposure conform with the findings of the present study.

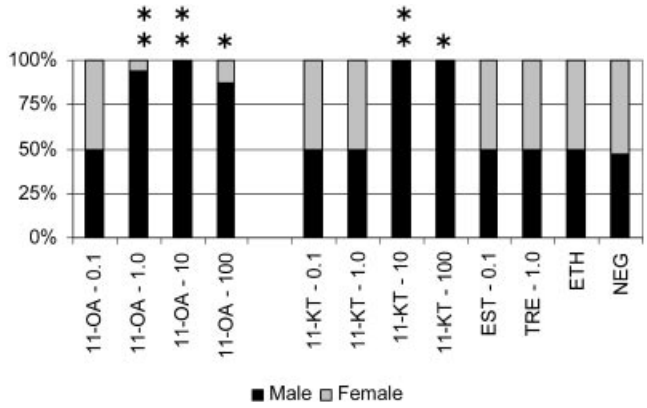

Fig. 1. Induction of papillary processes, a male-exclusive sex characteristic, on the anal fin in female medaka fish exposed to the mammalian 11-oxygenated cortisol metabolite 5α-androstan-3,11,17-trione (11-OA; 0.1–100 μg/L) and the major fish androgen 11-ketotestosterone (11-KT; 0.1–100 μg/L) for 14 to 21 d as indicated by the male/female proportion (%) in the exposure groups, which had been equal in all treatment groups at the beginning of exposure. Additional control and reference groups tested simultaneously include 17β-estradiol (EST; 0.1 μg/L), 17β-trenbolone (TRE; 1.0 μg/L), solvent ethanol control (ETH; 20 μg/L), and dilution water control (NEG). All concentrations nominal. Significance of differences between the exposure and the dilution water control groups in the ratio of male fish: $p < 0.05$, $p < 0.01$ (two-sided). Further details are given in Supplemental Data, Table S1.

Trenbolone

Other than expected, in the present study, 14 to 21 d of exposure of female medaka to 1.0 μg/L (nominal) 17β-trenbolone did not induce masculinization in the anal fin. In contrast, the range of 17β-trenbolone LOEC values for masculinization of secondary sex characteristics in females reported in the literature ranged from approximately 0.05 μg/L for medaka, to 0.5 μg/L for fathead minnow, to 5.0 μg/L in stickleback: 0.045 μg/L (nominal; solvent not reported; 21 d exposure [75]) and 0.5 μg/L (nominal; 0.365 μg/L measured; test medium solvent-free; 14–21 d [24]) for the induction of papillary processes on the anal fin in female medaka, 0.05 μg/L (nominal; 0.027 μg/L measured; 21 d; test medium solvent-free [23]) and 0.5 μg/L (nominal; 0.401 μg/L measured; test medium solvent free; 14–21 d [24]) for the induction of nuptial tubercles in female fathead minnow, and 5.0 μg/L (nominal; solvent control at 0.001% methanol; 14 d [38]) for the induction of spiggin production in female stickleback. There may be two, not mutually exclusive explanations for the apparently low androgenic potential of 17β-trenbolone observed in the present study, which will be discussed in the following paragraphs: atypical concentration response relationship and antagonistic solvent interaction.

Androgenic model test compounds

For the synthetic androgens used as model test compounds in standardized fish assays, there is increasing evidence that they not only do elicit androgenic effects at lower test concentrations but also elicit estrogenic effects at higher concentrations. Such atypical concentration–response relationships have been reported for methyltestosterone [14,80,81] and 17β-trenbolone, as discussed in detail by Ankley et al. [23] and Seki et al. [24]. For example, aquatic exposure to 1 μg/L (nominal) 17α-methyltestosterone increased plasma vitellogenin in male fathead minnow [25]; 17β-trenbolone at 41 μ/L (measured) increased 17β-estradiol and vitellogenin but decreased 11-KT levels in the plasma of male fathead minnow [23]).

Now, it appears that both methyltestosterone (as summarized by Cheshenko et al. [82]) and 17β-trenbolone [83] have the potential to increase gene expression of the cytochrome P450 gonadal aromatase (CYP19) metabolizing system in teleost fish, including medaka. It was concluded that the ''estrogenic effects of testosterone or α-methyltestosterone can be blocked by coexposure with aromatase inhibitor in fathead minnow (as summarized by Zerulla et al. [80]) or medaka [84], indicating the involvement of aromatase CYP19 in the process of paradoxical feminization'' [82]. In addition, it was pointed out that ''comparable up-regulation of ovarian CYP19 expression also has been observed after the exposure of medaka to an aromatase inhibitor, fadrozole, which suggests a similar compensatory mechanism in response to decreased endogenous estrogen [85] caused by aromatizable androgenic substances. However, the mode of action of trenbolone on CYP19 expression still needs to be investigated by assessing the levels of endogenous proteins''

[83]. In this context, it also has to be taken into consideration that carrier solvents may interact with the P450 metabolizing system in different ways, resulting in either down- or up-regulation of gene expression and enzyme activities, as reviewed by Hutchison et al. [86]; genotypic sex of the test organisms [75,87], and interspecific differences resulting from different lines of androgen receptor evolution in teleost fish (''genomic and syntenic analyses in addition to lack of PCR amplification show that one of the duplicated copies, AR-B, was lost in several basal Clupeocephala such as Cypriniformes, including the model species zebrafish'' [88]). Finally, reproductive phase and developmental stage of the fish may influence variation in the effect variables [46,48,49,89].

Other than testosterone, 11-KT is not converted into an estrogen [14], and we are not aware of any atypical concentration–response relationship reported to date. Therefore, we suggest including 11-KT as a primary model test compound in endocrine-disruptors testing protocols with fish.

Modes of action

Little is known about the mechanisms underlying fish androgen hormones actions or their interactions with each other and other modulating factors, including hormonal receptor-mediated mechanisms, metabolism, and transport (such as teleost sex steroid binding proteins [42]). For many fish species, plasma levels of 11-KT were found to exceed those of other androgens in males while remaining low in females, and 11-KT was considered to be the major influence on many androgen-dependent changes in the male reproductive state [14,42,90]. Now, information on the variability of androgen receptors in teleost fish is rapidly increasing (two receptor types in multiple isoforms have been described [88,90,91]). Interestingly, most of these androgen receptors displayed high testosterone affinity but significantly lower 11-KT affinity. Notably, the zebrafish androgen receptor showed high binding affinity to 11-KT, perhaps indicating that in other teleosts the AR-B, lost in zebrafish, plays a major role in 11-KT modes of action [45,92,93], and, only recently, an ''AR subtype (ARβ2) that is preferentially activated by 11-KT compared with testosterone has been identified in the male stickle-back'' [90,91]. Still, ''the mechanism by which 11-KT exhibits such high potency remains unclear,'' and ''elucidation of the KT signaling mechanism in teleosts clearly requires further research'' [91].

Gene expression profiling is adding further urgently needed information. It was found that treatment of Qurt medaka with 100 μg/L 11-KT caused significant differential expression of at least 578 genes (using the oligonucleotide microarray including 9,379 probes for EDC-affected genes, medaka cDNAs, sequences from the medaka genome project, and the UniGene database [47]). Finally, 11-KT was confirmed to play an important role in spermatogenesis [94,95] and the regulation of early oocyte growth ''with potential consequences for the fecundity process'' [46,48].

Preliminary risk characterization

Overall, LOEC values for aquatic exposure of medaka range from 2 μg/L 11-KT [58] to 10 μg/L 11-KT and 1.0 μg/L 11-OA (present study). Taking into account that these results are based on nominal concentrations only, and larger sample sizes would have increased the power of these analyses (a frequent handicap in endocrine-disruptors research with fish [96]), it is suggested that the actual LOEC values for these 11-oxygenated $C_{19}H_xO_3$ steroids at least fall within the range of the glucocorticoid levels detected in the aquatic environment. In sewage treatment plant effluents, cortisone levels reached approximately 10 μg/L (11–243 ng dexamethasone-equivalents/L [11] equal to 366.67–8,100 ng/L cortisone) and in surface waters approximately 100 ng/L cortisone (cortisol and cortisone: 0.50 and 0.26 ng/L; study areas in China [10,12]; 0.75–2.7 and 0.41–2.4 ng dexamethasone-equivalents/L; study areas in The Netherlands [11,67]; equal to 25.00–90.00 and 13.67–80.00 ng/L cortisone [corticosteroid converter http://www.globalrph.com/steroid.cgi]). Also for runoff and surface water from livestock farming, estimated levels rank at the nanograsms per liter level for corticosteroids and their metabolites from cattle excrement [present study, see Introduction]). Hence, based on the currently available database, environmental risk from mammalian cortisol metabolites to fish cannot be excluded.

Final considerations

In the present study, the hypothesis was confirmed that the mammalian cortisol metabolite 11-OA has the potential to act similarly to the 11-oxygenated fish androgen 11-KT in masculinizing external sex characteristics in female fish. Furthermore, the present study supports 11-KT as a strong natural androgenic model test compound in standardized OECD fish assays for endocrine-active substances with medaka, an atypical concentration–response relationship for the strong synthetic androgen 17β-trenbolone and its reevaluation as an androgenic test compound, solvents as possible confounding factors, development and validation of solvent-reduced or -free application procedures for endocrine-disruptors testing, existing environmental risk from 11-oxygenated $C_{19}H_xO_3$ steroids and mammalian corticosteroid metabolites, and livestock farming as an important source of corticosteroid endocrine disruptors. Hence, results from this pilot study point out an urgent need for a comprehensive assessment of both exposure and effects for mammalian glucocorticoids and their metabolites in fish.

SUPPLEMENTAL DATA

Table S1. Effects of the mammalian 11-oxygenated cortisol metabolite 5α-androstan-3,11,17-trione and the major fish androgen 11-ketotestosterone on external sex characteristics in the anal and dorsal fin of adult medaka. (105 KB DOC).

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REFERENCES

1. Muller M, Rabenoelina F, Balaguer P, Patureau D, Lemenach K, Budzinski H, Barcelo D, Lopez de Alda M, Kuster M, Delgenes J-P, Hernandez-Raquet G. 2008. Chemical and biological analysis of endocrine-disrupting hormones and estrogenic activity in an advanced sewage treatment plant. *Environ Toxicol Chem* 27:1649–1658.
2. Lange IG, Daxenberger A, Schiffer B, Witters H, Ibarreta D, Meyer HHD. 2002. Sex hormones originating from different livestock production systems: Fate and potential disrupting activity in the environment. *Anal Chim Acta* 473:27–37.
3. Burnison BK, Hartmann A, Lister A, Servos MR, Ternes T, Van Der Kraak G. 2003. A toxicity identification evaluation approach to studying estrogenic substances in hog manure and agricultural runoff. *Environ Toxicol Chem* 22:2243–2250.
4. Lorenzen A, Hendel JG, Conn KL, Bittman S, Kwabiah AB, Lazarovitz G, Masse D, McAllister TA, Topp E. 2004. Survey of hormone activities in municipal biosolids and animal manures. *Environ Toxicol* 19:216–225.

5. Bursch W, Fuerhacker M, Gemeiner M, Grillitsch B, Jungbauer A, Kreuzinger N, Moestl E, Scharf S, Schmid E, Skutan S, Walter I. 2004. Endocrine disrupters in the aquatic environment: The Austrian approach. *Water Sci Technol* 50:293–300.
6. Smital T. 2008. Acute and chronic effects of emerging contaminants. In Barceló D, Petrovic M, eds, *Emerging Contaminants From Industrial and Municipal Waste. Occurrence, Analysis and Effects. The Handbook of Environmental Chemistry*, Vol 5S/1. Springer, Berlin, Germany, pp 105–142.
7. Bjoerkblom C, Hoegfors E, Salste L, Bergelin E, Olsson P-E, Katsiadaki I, Wiklund T. 2009. Estrogenic and androgenic effects of municipal wastewater effluent on reproductive endpoint biomarkers in three-spined stickleback (*Gasterosteus aculeatus*). *Environ Toxicol Chem* 28:1063–1071.
8. Sowers AD, Gaworecki KM, Mills MA, Roberts AP, Klaine SJ. 2009. Developmental effects of a municipal wastewater effluent on two generations of the fathead minnow. *Pimephales promelas*. *Aquat Toxicol* 95:173–181.
9. Ingerslev F, Vaclavik E, Halling-Sorensen B. 2003. Pharmaceuticals and personal care products—A source of endocrine disruption in the environment? *Pure Appl Chem* 75:1881–1893.
10. Chang H, Hu J, Shao B. 2007. Occurrence of natural and synthetic glucocorticoids in sewage treatment plants and receiving river waters. *Environ Sci Technol* 41:3462–3468.
11. Van der Linden SC, Heringa MB, Man HY, Sonneveld E, Puijker LM, Brouwer A, Van der Burg B. 2008. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. *Environ Sci Technol* 42:5814–5820.
12. Chang H, Wan Y, Hu J. 2009. Determination and source apportionment of five classes of steroid hormones in urban rivers. *Environ Sci Technol* 43:7691–7698.
13. Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G. 2002. *Global Assessment of the State-of-the-Science of Endocrine Disrupters*. World Health Organization, International Programme on, Chemical Safety (IPCS), Geneva, Switzerland.
14. Organisation for Economic Co-operation and Development. 2004. Detailed review paper on fish screening assays for the detection of endocrine active substances. Paris, France.
15. Devillers J. 2009. *Endocrine Disruption Modeling*. CRC, New York, NY, USA.
16. Kendall R, Dickerson R, Giesy J, Suk W. 1998. *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*. SETAC, Pensacola, FL, USA.
17. Kime DE. 1998. *Endocrine Disruption in Fish*. Kluwer Academic, Norwell, MA, USA.
18. Katsiadaki I, Scott AP, Hurst MR, Matthiessen P, Mayer I. 2002. Detection of environmental androgens: a novel method based on enzyme-linked immunosorbant assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. *Environ Toxicol Chem* 21:1946–1954.
19. Sebire M, Katsiadaki I, Scott AP. 2007. Non-invasive measurement of 11-ketotestosterone, androstenedione and cortisol in male three-spined stickleback (*Gasterosteus aculeatus*). *Gen Comp Endocrinol* 152:30–38.
20. Daxenberger A. 2002. Pollutants with androgen-disrupting potency. *Eur J Lipid Sci Technol* 104:124–130.
21. Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. 2009. Endocrine-disrupting chemicals: An Endocrine Society Scientific Statement. *Endocr Rev* 30:293–342.
22. Weiss JM, Hamers T, Thomas KV, van der Linden S, Leonards PE, Lamoree MH. 2009. Masking effect of anti-androgens on androgenic activity in European river sediment unveiled by effect-directed analysis. *Anal Bioanal Chem* 394:1385–1397.
23. Ankley GT, Jensen KM, Makynen EA, Kahl MD, Korte JJ, Hornung MW, Henry TR, Denny JS, Leino RL, Wilson VS, Cardon MC, Hartig PC, Gray LE. 2003. Effects of the androgenic growth promoter 17-β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environ Toxicol Chem* 22:1350–1360.
24. Seki M, Fujishima S, Nozaka T, Maeda M, Kobayashi K. 2006. Comparison of response to 17β-estradiol and 17β-trenbolone among three small fish species. *Environ Toxicol Chem* 25:2742–2752.
25. Pawlowski S, Sauer A, Shears JA, Tyler CR, Braunbeck T. 2004. Androgenic and estrogenic effects of the synthetic androgen 17alpha-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. *Aquat Toxicol* 68:277–291.
26. Barry TP, Marwah A, Marwah P. 2007. Stability of 17α-methyltestosterone in fish feed. *Aquaculture* 271:523–529.
27. McAllister BG, Kime DE. 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). *Aquat Toxicol* 65:309–316.
28. Shimasaki Y, Kitano T, Oshima Y, Inoue S, Imada N, Honjo T. 2003. Tributyltin causes masculinization in fish. *Environ Toxicol Chem* 22:141–144.
29. Santos MM, Micael J, Carvalho AP, Morabito R, Booy P, Massanisso P, Lamoree M, Reis-Henriques MA. 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. *Comp Biochem Physiol C Toxicol Pharmacol* 142:151–155.
30. U.S. Environmental Protection Agency. 2009. *Targeted National Sewage Sludge Survey Overview Report*. EPA-822-R- 08-014. Washington, DC.
31. Larsson DGJ, Adolfsson-Erici M, Thomas P. 2006. Characterization of putative ligands for fish gonadal androgen receptor in a pulp mill effluent. *Environ Toxicol Chem* 25:419–427.
32. Wartman CA, Hogan NS, Hewitt LM, McMaster ME, Landman MJ, Taylor S, Kovacs TG, van den Heuvel MR. 2009. Androgenic effects of a Canadian bleached kraft pulp and paper effluent as assessed using threespine stickleback (*Gasterosteus aculeatus*). *Aquat Toxicol* 92:131–139.
33. Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, von Guten U, Wehrli B. 2006. The challange of micropollutants in aquatic systems. *Science* 313:1072–1077.
34. Petrovic M, Radjenovic J, Postigo C, Kuster M, Farre M, de Alda ML, Barcelo D. 2008. Emerging contaminants in waste waters: sources and occurrence. In Barcelo D, Petrovic M, eds, *Emerging Contaminants From Industrial and Municipial Waste. The Handbook of Environmental Chemistry*. Springer, Berlin, Germany, pp 1–35.
35. Sumpter JP. 2008. The ecotoxicology of hormonally active micropollutants. *Water Sci Technol* 57:125–130.
36. Organisation for Economic Co-operation and Development. 2009. OECD Guidelines for the Testing of Chemicals. Endocrine Disrupter Testing and Assessment. Peer Review Package for the 21-day Fish Endocrine Screening Assay and Peer Review Package for the Androgenised Female Stickleback Screening Assay. Paris, France.
37. U.S. Environmental Protection Agency. 2009. Endocrine Disruptor Screening Program. Assay Development and Validation. Washington, DC.
38. Allen YT, Katsiadaki I, Pottinger TG, Jolly C, Matthiessen P, Mayer I, Smith A, Scott AP, Eccles P, Sanders MB, Pulman KG, Feist S. 2008. Intercalibration exercise using a stickleback endocrine disrupter screening assay. *Environ Toxicol Chem* 27:404–412.
39. Organisation for Economic Co-operation and Development. 2009. 21-Day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition. Guideline 230. Paris, France.
40. Organisation for Economic Co-operation and Development. 2009. Androgenised Female Stickleback Screen (AFSS). Preliminary Draft Test Guideline. Paris, France.
41. Hishida T-O, Kawamoto N. 1970. Androgenic and male-inducing effects of 11-ketotestosterone on a teleost, the medaka (*Oryzias latipes*). *J Exp Zool* 173:279–283.
42. Borg B. 1994. Androgens in teleost fishes. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 109:219–245.
43. Lokman PM, Harris B, Makoto Kusakabe M, Kime DE, Schulz RW, Adachi S, Young G. 2002. 11-Oxygenated androgens in female teleosts: Prevalence, abundance, and life history implications. *Gen Comp Endocrinol* 129:1–12.
44. Ankley GT, Johnson RD. 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *ILAR Inst Lab Anim Res J* 45:469–483.
45. Jorgensen A, Andersen O, Bjerregaard P, Rasmussen LJ. 2007. Identification and characterisation of an androgen receptor from zebrafish *Danio rerio*. *Comp Biochem Physiol C Toxicol Pharmacol* 146:561–568.
46. Kortner TM, Rocha E, Silva P, Castro LFC, Arukwe A. 2008. Genomic approach in evaluating the role of androgens on the growth of Atlantic cod (*Gadus morhua*) previtellogenic oocytes. *Comp Biochem Physiol D Genom Proteom* 3:205–218.
47. Leon A, Wu P-S, Hall LC, Johnson ML, Teh SJ. 2008. Global gene expression profiling of androgen disruption in Qurt strain medaka. *Environ Sci Technol* 42:962–969.
48. Kortner et al. 2009. Kortner T, Eduardo R, Arukwe A. 2009.Androgenic modulation of early growth of Atlantic cod (*Gadus morhua* L.) previtellogenic oocytes and zona radiata-related genes. *J Toxicol Environ Health A* 72:184–195.

49. Leon A, Teh SJ, Hall LC, The FC. 2007. Androgen disruption of early development in Qurt strain medaka (*Oryzias latipes*). *Aquat Toxicol* 82:195–203.
50. Bhandari RK, Alam MA, Soyano K, Nakamura M. 2006. Induction of female-to-male sex change in the honeycomb grouper (*Epinephelus merra*) by 11-ketotestosterone treatments. *Zool Sci* 23:65–69.
51. Borg B, Antonopoulou E, Andersson E, Carlberg T, Mayer I. 1993. Effectiveness of several androgens in stimulating kidney hypertrophy, a secondary sexual character, in castrated male three-spined sticklebacks, *Gasterosteus aculeatus*. *Can J Zool* 71:2327–2329.
52. Angus RA, Mcnatt HB, Howell WM, Peoples SD. 2001. Gonopodium development in normal male and 11-ketotestosterone-treated female mosquitofish (*Gambusia affinis*): A quantitative study using computer image analysis. *Gen Comp Endocrinol* 123:222–234.
53. Scott AP, Hirschenhauser K, Bender N, Oliveira R, Earley RL, Sebire M, Ellis T, Pavlidis M, Hubbard PC, Huertas M, Canario A. 2008. Non-invasive measurement of steroids in fish-holding water: Important considerations when applying the procedure to behaviour studies. *Behaviour* 145:1307–1328.
54. Hirschenhauser K, Canario AVM, Ros AFH, Taborsky M, Oliveira RF. 2008. Social context may affect urinary excretion of 11-ketotestosterone in African cichlids. *Behaviour* 145:1367–1388.
55. Egami N. 1975. Secondary sexual characters. In Yamamoto T, ed, *Medaka (Killifish): Biology and Strains*. Keigaku, Tokyo, Japan, pp 17–29.
56. Edmunds JSG, McCarthy RA, Ramsdell JS. 2000. Permanent and functional male-to-female sex reversal in d-rR strain Medaka (*Oryzias latipes*) following egg microinjection of o,p′-DDT. *Environ Health Perspect* 108:219–224.
57. Kinoshita M, Murata K, Naruse K, Tanaka M. 2009. *Medaka: Biology, Management, and Experimental Protocols*. Wiley-Blackwell, Hoboken, NJ, USA.
58. Asahina K, Urabe A, Sakai T, Hirose H, Hibiya T. 1989. Effects of various androgens on the formation of papillary processes on the anal fin rays in the female medaka. *Oryzias latipes, Nippon Suisan Gakkaishi* 55:1871–1871.
59. Fox GA. 2004. Chemical communication threatened by endocrine-disrupting chemicals. *Environ Health Perspect* 112:648–653.
60. Claessens F, Denayer S, Van Tilborgh N, Kerkhofs S, Helsen C, Haelens A. 2008. Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. *Nucl Recept Signal* 6:1–13.
61. van den Berg M, Sanderson T, Kurihara N, Katayama A. 2003. Role of metabolism in the endocrine-disrupting effects of chemicals in aquatic and terrestrial systems. *Pure Appl Chem* 75:1917–1932.
62. Carson JD, Jenkins RL, Wilson EM, Howell WM, Moore R. 2008. Naturally occurring progesterone in loblolly pine (*Pinus taeda* L.): A major steroid precursor of environmental androgens. *Environ Toxicol Chem* 27:1273–1278.
63. Jenkins R, Angus RA, McNatt H, Howell WM, Kemppainen JA, Kirk M, Wilson EM. 2001. Identification of androstenedione in a river containing paper mill effluent. *Environ Toxicol Chem* 20:1325–1331.
64. Larsson DGJ, Förlin L. 2002. Male-biased sex ratios of fish embryos near a pulp mill: temporary recovery after a short-term shutdown. *Environ Health Perspect* 110:739–742.
65. Jenkins RL, Wilson EM, Angus RA, Howell WM, 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 mill effluent. *Environ Health Perspect* 112:1508–1511.
66. Hewitt LM, Kovac TG, Dube MG, MacLatchy DL, Martel PH, McMaster ME, Paice MG, Parrott JL, van den Heuvel MR, Van Dkraak GJ. 2008. Altered reproduction in fish exposed to pulp and paper mill effluents: Roles of individual compounds and mill operating conditions. *Environ Toxicol Chem* 27:682–697.
67. Schriks M, Heringa MB, Sander C, van der Linden SC. 2009. Temporal variation in multiple hormonal activities of surface waters located in the Dutch part of the Rhine basin. Association of Rhine Water Works, Amsterdam, The Netherlands.
68. Möstl E, Messmann S, Bagu E, Robia C, Palme R. 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. *J Vet Med A* 46:621–632.
69. Palme R, Robia C, Baumgartner W, Möstl E. 2000. Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. *Vet Rec* 146:8–9.
70. Möstl E, Maggs JL, Schrötter G, Besenfelder U, Palme R. 2002. Measurement of cortisol metabolites in faeces of ruminants. *Vet Res Commun* 26:127–139.
71. Lexen E, ElBahr SM, Sommerfeld-Stur I, Palme R, Möstl E. 2008. Monitoring the adrenocortical response to disturbances in sheep by measuring glucocorticoid metabolites in the faeces. *Vet Med Austria* 95: 64–71.
72. Johnson AC, Williams RJ, Matthiessen P. 2006. The potential steroid hormone contribution of farm animals to freshwaters, the United Kingdom as a case study. *Sci Total Environ* 362:166–178.
73. Cerone-McLernon AM, Winter J, Mosbach EH, Bokkenheuser VD. 1981. Side-chain cleavage of cortisol by fecal flora. *Biochim Biophys Acta Lipids Lipid Metabol* 666:341–347.
74. Yamamoto T. 1975. Intoductory Remarks on the Medaka. In Yamamoto T, ed, *Medaka (Killifish): Biology and Strains*. Keigaku, Tokyo, Japan, pp 1–16.
75. Flynn K, Lothenbach D, Hammermeister D, Haselman J, Sheedy B, Whiteman F, Johnson R. 2005. Comparative sensitivity of androgenic effects in medaka (*Oryzias latipes*) following exposure to 17β-trenbolone. *Abstracts*, SETAC 26th Annual Meeting in North America, Baltimore, MD, USA, November 13–15, 2005.
76. Organisation for Economic Co-operation and Development. 2006. Current approaches in the statistical analysis of ecotoxicity data. A guidance to application. Guideline 54. Paris, France.
77. Scott AP, Pinillos ML, Huertas M. 2005. The rate of uptake of sex steroids from water by *Tinca tinca* is influenced by their affinity for sex steroid binding protein in plasma. *J Fish Biol* 67:182–200.
78. Yamamoto T. 1969. Sex differentiation. In Hoar WS, Randall DJ, eds, *Fish Physiology III*. Academic, New York, NY, USA, pp 117–175.
79. Zentel HJ. 1988. Experimentelle Untersuchungen zur Geschlechtsdifferenzierung beim Guppy *(Poecilia reticulata)*. PhD thesis, University of Mainz, Mainz, Germany.
80. Zerulla M, Laenge R, Steger-Hartmann T, Panter G, Hutchinson T, Dietrich DR. 2002. Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). *Toxicol Lett* 131:51–63.
81. Andersen L, Goto-Kazato R, Trant JM, Nash JP, Korsgaard B, Bjerregaard P. 2006. Short-term exposure to low concentrations of the synthetic androgen methyltestosterone affects vitellogenin and steroid levels in adult male zebrafish (*Danio rerio*). *Aquat Toxicol* 76:343–352.
82. Cheshenko K, Pakdel F, Segner H, Kah O, Eggen RIL. 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *Gen Comp Endocrinol* 155:31–62.
83. Park J, Tompsett A, Zhang X, Newsted JL, Jones PD, Au D, Kong R, Wu R, Giesy JP, Hecker M. 2009. Advanced fluorescence in situ hybridization to localize and quantify gene expression in Japenese medaka (*Oryzias latipes*) exposed to endocrine-disrupting compounds. *Environ Toxicol Chem* 28:1951–1962.
84. Iwamatsu T, Kobayashi H, Sagegami R, Shuo T. 2006. Testosterone content of developing eggs and sex reversal in the medaka (*Oryzias latipes*). *Gen Comp Endocrinol* 145:67–74.
85. Park JW, Tompsett AR, Zhang X, Newsted JL, Jones PD, Au DWT, Kong RYC, Wu RSS, Giesy JP, Hecker M. 2008. Fluorescence in situ hybridization techniques (FISH) to detect changes in CYP19a gene expression of Japanese medaka (*Oryzias latipes*). *Toxicol Appl Pharmacol* 232:226–235.
86. Hutchinson TH, Shillabeer N, Winter MJ, Pickford DB. 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. *Aquat Toxicol* 76:69–92.
87. Grim KC, Wolfe M, Hawkins W, Johnson R, Wolf J. 2007. Intersex in Japanese medaka (*Oryzias latipes*) used as negative controls in toxicologic bioassays: a review of 54 cases from 41 studies. *Environ Toxicol Chem* 26:1636–1643.
88. Douard V, Brunet F, Boussau B, Ahrens-Fath I, Vlaeminck-Guillem V, Haendler B, Laudet V, Guiguen Y. 2008. The fate of the duplicated androgen receptor in fishes: A late neofunctionalization event? *BMC Evol Biol* 8:336.
89. Rolland AD, Lareyre J-J, Goupil A-S, Montfort J, Ricordel M-J, Esquerre D, Hugot K, Houlgatte R, Chalmel F, Le Gac F. 2009. Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. *BMC Genom* 10:546.
90. Harbott LK, Burmeister SS, White RB, Vagell M, Fernald RD. 2007. Androgen receptors in a Cichlid fish, *Astatotilapia burtoni*: Structure, localization, and expression levels. *J Comp Neurol* 504: 57–573.

91. Olsson PE, Berg AH, von Hofsten J, Grahn B, Hellqvist A, Larsson A, Karlsson J, Modig C, Borg B, Thomas P. 2005. Molecular cloning and characterization of a nuclear androgen receptor activated by 11-ketotestosterone. *Reprod Biol Endocrinol* 3:37.
92. Hossain MS, Larsson A, Scherbak N, Olsson P-E, Orban L. 2008. Zebrafish androgen receptor: isolation, molecular, and biochemical characterization. *Biol Reprod* 78:361–369.
93. de Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J. 2008. Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction* 136:225–234.
94. Cavaco JE, Bogerd J, Goos H, Schulz RW. 2001. Testosterone inhibits 11-ketotestosterone-induced spermatogenesis in African catfish (*Clarias gariepinus*). *Biol Reprod* 65:1807–1812.
95. Miura T, Miura C, Konda Y, Kohei Yamauchi K. 2002. Spermatogenesis-preventing substance in Japanese eel. *Development* 129:2689–2697.
96. Bosker T, Munkittrick KR, MacLatchy D. 2009. Challenges in current adult fish laboratory reproductive tests: Suggestions for refinement using a mummichog (*Fundulus heteroclitus*) case study. *Environ Toxicol Chem* 28:2386–2396.