Functional genomics and sexual differentiation in amphibians

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Abstract

In *Xenopus laevis* the basic mechanisms underlying sexual differentiation were investigated by determining time courses of sexual steroids and their corresponding receptors during complete larval development from egg to juveniles. Androgens as well as estradiol (E2) are derived from maternal origin and accumulate in hatching tadpoles. Sexual steroid contents decreased rapidly after hatching and rose again at the end of metamorphosis indicating endogenous production. In parallel the mRNA expression for corresponding androgen (AR) and estrogen receptors (ER) was measured by means of semiquantitative RT-PCR. Both receptor mRNAs increased dramatically just after hatching and decreased only moderately until end of metamorphosis. In female juveniles E2 and ER-mRNA levels were higher compared with males. Treatment by exogenous E2 elevated both, ER- and AR-mRNA, indicating stimulatory functions of E2 for gene expression of both receptors. Effects on sexual differentiation during larval development were achieved by treatment with E2 and the antiandrogen cyproterone acetate both causing feminization, the antiestrogen tamoxifen resulting in neutralization, and the androgens, methyltestosterone and dihydrotestosterone, but not testosterone, leading to masculinization. The data presented are in accordance with further recent findings and suggest a new hypothesis for functional genomics in sexual differentiation of amphibians.

Keywords: Amphibian; *Xenopus laevis*; Sexual differentiation; Estradiol; Androgens; Estrogen receptor; Androgen receptor; Functional genomics

1. Introduction

In all vertebrate species sexual differentiation is regulated during development by a complex interplay of functional genomics triggered by endogenous as well as exogenous factors. Amphibians are the classical models in endocrinology for the investigation of effects on sexual differentiation because of their obvious response to severe treatments by sex reversal (Witschi and Allison, 1950; Witschi, 1971; Gallien, 1974; Rastogi and Chieffi, 1975; Kelley, 1996; Hayes, 1998; Wallace et al., 1999; Kloas et al., 1999; Kloas, 2002). There is growing interest in understanding basic mechanisms underlying sexual differentiation of amphibians because it is now well established that amphibians are very sensitive to several environmental factors including endocrine disrupting compounds (EDC) and it is of major concern that EDC may contribute markedly to the worldwide decline of amphibians (Carey and Bryant, 1995; Stebbins and Cohen, 1995). EDC are known to interfere with the endocrine systems of wildlife.
and humans affecting mainly reproductive biology, including sexual differentiation and the thyroid system (Colborn et al., 1993). However, EDC can interact principally at multiple targets of the endocrine system, which may lead to adverse effects on sexual differentiation in amphibians (Kloas, 2002), and therefore more basic knowledge is needed to understand the molecular mechanisms how this might be accomplished.

The sexual steroids, estrogens and androgens, have major functions by complex interplay with other endocrine factors, which facilitates normal sexual differentiation, development, and maintenance of sexual functions by production of fertile spermatozoa and oocytes, concomitant with corresponding sexual behavior during reproduction. The significance of sexual steroids depend on the presence of their corresponding receptors needed to induce specific cell signaling pathways via genomic functions. For instance estradiol (E2) is known to generate autoinduction of the estrogen receptor (ER) potentiating E2 specific genomic activation (Rabelo and Tata, 1993). Despite the importance of such information no data were available to indicate time courses of sexual steroids and their corresponding estrogen and androgen receptors during complete larval development of amphibians including sexual differentiation. The aim of the present study, using the amphibian *Xenopus laevis* as model, was to determine the contents of sexual steroids, estradiol (E2) and androgens, by radioimmunoassays and the presence of estrogen (ER) and androgen receptors (AR) by assaying their corresponding mRNAs using semiquantitative RT-PCR. In addition, to demonstrate potential genomic effects of E2 and androgens on gene expression for ER and AR short term experiments were performed with juvenile *X. laevis* to determine potential changes of mRNAs for ER and AR. Furthermore, in vivo treatments by estrogenic, antiestrogenic, androgenic, and antiandrogenic compounds during larval development were done to assess their impact on sexual development resembling in a recent hypothesis for sexual differentiation in amphibians.

2. Materials and methods

2.1. Animals and treatments

All experiments were carried out with *Xenopus laevis* tadpoles or juveniles reared from the animal stock of the Department of Inland Fisheries, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin. Spawning of adult *X. laevis* was induced as described elsewhere (Kloas et al., 1997) by injecting human chorionic gonadotropin (HCG, Sigma, Deisenhofen). Eggs and developing tadpoles were kept in 50-liter tanks containing well-aerated artificial tap water at 23 °C under a 12:12-h light-dark cycle (lights on 06:00–18:00 h). Tadpoles were fed three times a week with Sera micron (Rheinsberg, Germany). Juvenile animals were kept similarly without aeration and fed twice a week with a diet of minced pork heart.

2.1.1. Sampling for time courses of sexual steroids and mRNA expression during development

*Xenopus laevis* tadpoles developed from stage 1 to 35 during 2–3 days in the egg shell and eggs were collected at day 2 at developmental stages of 20 ± 5 (Nieuwkoop and Faber, 1994). All experimental tadpoles were reared from spawns of three couples in parallel. Up to stage 48 animals were pooled to get enough mass [approx. 200 mg body weight (b.w.)] for assaying steroid and mRNA contents. Animals were weighed, anesthetized by cooling on ice, and killed at stages <48 by placing on dry ice while beginning at stage 48 they were killed first by pithing the spinal cord before placing on dry ice. All samples were weighed and kept at −80 °C until steroid or total RNA extractions. Sampling was performed for steroid and mRNA determinations, respectively, as following: stage 20, 38, 41, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, and juveniles 2 months after completion of metamorphosis. Six samples were taken for each stage until stage 54, while starting at stage 56 animals were rapidly investigated by gross morphology of gonads using a binocular microscope and subdivided into males and females, each grouping consisting of six individuals per stage.

2.1.2. Induction of estrogen and androgen receptor mRNA by in vivo treatment.

Juvenile *Xenopus* (approx. 1 month after metamorphosis) were transferred randomly into six 5-l aquaria containing artificial tap water and reared as described above. Each aquarium contained 20 animals and all treatments were set up twice in parallel lasting one week. Groups consisted of controls, receiving the same amounts of 96% ethanol used as solvent for E2 and testosterone (T) treatment. E2 and T were added at 10⁻⁸ M.
Addition of freshly prepared substances or solvent started on Monday and was renewed after water exchange on Wednesday and Friday. Experiments were terminated at the following Monday by killing the animals, determining their sex, and samples were further processed for determinations of ER and AR-mRNA as described below.

2.1.3. In vivo treatment and sexual differentiation during larval development.

At the developmental stage 40 (3 days after hatching), tadpoles were transferred into 16 5-l aquaria containing well-aerated tap water at 23 °C and reared as described above. The experimental setup divided one batch of tadpoles into eight groups each consisting of 50 treated animals subdivided on two aquaria each containing 25 tadpoles. The groups consisted of control, the estrogen E2, the antiestrogen tamoxifen, the androgens, T, methyltestosterone (MT), and dihydrotestosterone (DHT), and the antiandrogen cyproterone acetate (all from Sigma, Deisenhofen, Germany). All compounds were added at a nominal concentration of 10^{-8} M and in addition E2 was given at 10^{-7} M. Addition of freshly prepared substances followed water exchange every Monday, Wednesday, and Friday until metamorphosis was accomplished in approximately 90% of all animals. During the whole experiment which took approximately 12 weeks size and developmental stage of controls and treated groups were estimated roughly every second week to look for obvious differences in growth, development, and survival rate. Animals were killed just after completing metamorphosis by immersion in MS 222 at 3 g/l and differentiation into male or female gonads was assayed by gross morphology using a binocular microscope at a magnification of 40× as described elsewhere (Kloas et al., 1999).

2.2. Determination of sexual steroids

Steroid extraction was accomplished at the day following sampling and done in glass vials by homogenizing samples with an Ultra-Turrax in 1 ml distilled water, adding 6 ml dichloromethane, and shaking on a horizontal shaker for 1 h. The aqueous phase containing tissue contents was frozen by putting samples into a deep freezer at −80 °C and the steroid containing liquid organic phase was collected into another glass vial and dried at room temperature. The dried steroids were kept again at −80 °C until steroid determination. Steroids were redissolved in 1 ml 5% ethanol and estradiol (E2) and androgens (T and DHT), were assayed in parallel by radioimmunoassays using 200 µl of redissolved steroids in duplicates for E2 and androgen assessments. The recovery rates of both E2 and androgens were determined by adding [³H]E2, [³H]T, and [³H]DHT (all Amersham, Braunschweig, Germany) using 10 samples each and were 65±9% for E2, 63±8% for T, and 67±8% for DHT, respectively. Determination of E2 was performed using a commercial available antibody against E2 (Sigma, Deisenhofen, Germany) in accordance to the detailed description of the supplier. The antibody against androgens (gift from Dr Maser-Gluth, Pharmacological Institute, University Heidelberg, Germany) is characterized by its equal high specificity for both androgens, T and DHT, whereas cross-reactivity with other steroids does not exceed more than 1%. Thus, our assay summarizes the contents of both androgens. The radioimmunoassay for androgen measurement was performed exactly as described for corticosterone determination (Kloas and Hanke, 1990) except using T (Sigma, Deisenhofen, Germany) for standards and [³H]T as tracer. Detection limits for E2 and androgens in samples were 2 pg and 5 pg, respectively. All steroid measurements are normalized to 1 g fresh body mass.

2.3. Determination of estrogen and androgen receptor-mRNAs by semiquantitative RT-PCR

The samples taken into Eppendorf vials were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was extracted using the phenolic reagent Trizol (GIBCO/BRL, Eggenstein, Germany). Trizol (750 µl) were added to one frozen sample at room temperature and the mass was minced with scissors, homogenized completely using an Ultra-Turrax, and incubated for 5 min. Addition of 150 µl chloroform (Roth, Karlsruhe, Germany) was done to separate lipophilic and hydrophilic phases by vortexing, incubating for 3 min, and centrifuging (12 000×g, 4 °C) for 15 min. RNA containing supernatant (300 µl) were taken and RNA was separated by adding 375 µl isopropanol (Roth, Karlsruhe, Germany), vortexing, incubating for 10 min, and centrifuging again for 10 min. Following aspiration of the supernatant, the RNA-pellet was resuspended in ice-cold 70% ethanol and again
resulting in a PCR product of 363 basepairs

The sequences of AR primers (Biometra, Göttin-
gen) were forward: 5′-GAGGAAATGTATTAGGCTGG-3′ and reverse: 5′-ACGGCTCATTTGTCGCTTTAC-3′. PCR reaction was performed as described above for ER cDNA.

The housekeeping gene elongation factor 1α (EF1α) of X. laevis (Dostal et al., 1994; Dosch et al., 1997) was used as internal standard by performing the PCR of ER, AR, and EF1α-cDNA from the same samples, each in duplicates. The PCR reaction for EF1α was done similarly using the primer sequences (Biometra) forward: 5′-TGCCAATTGTCGATCAGCC-3′ and reverse: 5′-TACTATTTAAACTCTGGCC-3′ resulting in an amplified sequence of 285 bp and amplification was achieved by 23 cycles.

PCR products were electrophoresed immediately or stored at −20 °C. Horizontal gel electrophoresis was carried out in 1.5% agarose gel in TAE-buffer (pH: 8.5, 20 mM Tris–HCl, 0.1% acetic acid, and 1 mM NaEDTA) at 60 mV for 45 min. Staining of cDNA was achieved by adding 0.1 μg/1 μl ethidium bromide per 10 ml liquid agarose gel. PCR products were visible under UV-light and their fluorescence caused by ethidium bromide staining was according to the quantity of bands of amplified cDNA. The fluorescent bands of cDNA were quantified by densitometric measurements using an image analyzer (M1-System, Ontario, Canada). Various contents of cDNA revealed over a wide densitometric range a linearity between quantity and optical density which allows semiquantitative measurement compared to a base value. The densitometric values of the cDNA for the house-keeping protein EF1α were used to serve as internal standard. Therefore the amounts of amplified ER and AR-cDNA were normalized for the differences obtained by the PCR products of EF1α.

The cDNAs of PCR products were extracted from agarosegels using a kit (QUIAquick, Quiagen, Hilden, Germany) and sequences were analyzed by automatic sequencing (Sequence Laboratories, Göttinigen, Germany) confirming the proposed sequences of the PCR products.

2.4. Statistical analyses

In order to evaluate statistical significance the non-parametric Mann–Whitney U-test was used. When needed, the presence of significant differences was pre-checked using of the Kruskal–Wallis test.
3. Results

3.1. Temporal changes in sexual steroids.

The time course of E2 (Fig. 1) during complete larval development demonstrated the presence of large amounts of \(2586 \pm 987\) pg/g b.w. already in eggs containing embryos of larval stage 20. During further development E2 accumulated in larvae leading to higher concentrations of approximately \(5948 \pm 2278\) pg/g b.w. in freshly hatched tadpoles of stage 38 followed by a dramatic decrease during premetamorphic stages. At stage 56, when differentiation in female and male gonads can be determined by gross morphological examination, a small increase could be observed in female individuals while males always had lower values being significant at stage 62 and in 2 months old juveniles when a clear elevation of E2 production is present in both sexes.

The androgens (T and DHT) were determined in parallel and showed a similar time course at the beginning (Fig. 2). The amounts of androgens in eggs start with \(2760 \pm 730\) pg/g b.w. and rose in freshly hatched larvae of stage 38 up to \(5421 \pm 2408\) pg/g b.w. The sharp decrease of androgens was less pronounced as for E2 and reached lowest levels at metamorphic stages when male individuals showed up higher values compared to female ones. Significant elevated androgen levels in males were found at stages 58 and 66.

3.2. Time courses of mRNAs for estrogen receptor (ER) and androgen receptor (AR)

Expression of ER-mRNA (Fig. 3) was not detectable in eggs and revealed very low levels in
freshly hatched tadpoles at stage 38 followed by a strong increase up to stage 54 which remained at such high levels during further development only in females while males had always lower values being significantly different only in 2 months old juveniles.

The development of a method to determine AR-mRNA by semiquantitative RT-PCR was successful demonstrating a clear band of amplified cDNA with a length of 363 bp and the house keeping gene EF1α as internal standard (Fig. 4). The pattern of AR-mRNA during larval development (Fig. 5) was very similar to that of ER-mRNA but the increase seemed to be faster and clear differences between both sexes could not be observed.

3.3. ER- and AR-mRNA after in vivo treatment by E2 and T

Short term exposure of 1-month-old juvenile *Xenopus* revealed that treatment with $10^{-8}$ M E2 elevated significantly both, ER- and AR-mRNA, while exposure to $10^{-8}$ M T was without any significant effect (Fig. 6).

3.4. Sexual differentiation during larval development

The results of in vivo treatment by estrogenic, antiestrogenic, androgenic, and antiandrogenic compounds are summarized in Fig. 7. Treatment
with the estrogen E2 caused significant feminization being highly significant already at $10^{-8}$ M ($P < 0.01$) and nearly complete at $10^{-7}$ M ($P < 0.001$). The antiestrogen tamoxifen at $10^{-8}$ M caused no change in sex ratio but the gonads were always less developed suggesting a non-functioning state due to underdevelopment. The androgens used, T, MT, and DHT, showed varying effects at $10^{-8}$ M. T was not efficient to change sex ratio, while MT and DHT demonstrated masculinization in a similar manner. The antiandrogen cyproterone acetate, however, resulted in feminization like estrogenic compounds.

4. Discussion

In general, four principal modes of action may have a marked impact on reproductive biology via estrogenic, antiestrogenic, androgenic, and antiandrogenic effects in vertebrates. The complex humoral interplay of hypothalamus–pituitary–gonad axes regulates the levels of the sexual steroids, estrogens and androgens, which are mainly responsible for sexual differentiation and maturation of fertile spermatozoa and oocytes. In general, estrogenic compounds lead to feminization, antiestrogenic substances neutralize sexual differentiation, androgenic agents cause masculinization, while antiandrogenic compounds have feminizing effects (Döhler and New, 1989).

Amphibians are known to change their sex ratio during sexual differentiation following treatments with sexual steroids during larval development independent from their genotype. However, it is still a matter of debate whether such general characterization of modes of action as discussed by Döhler and New (1989) for vertebrates is applicable to amphibians. The results of some experiments seem to be contradictory concerning sexual differentiation of amphibians (Gallien, 1974; Hayes, 1998; Wallace et al., 1999). Only estrogens led always to a clear feminization, whereas masculinization by the androgen T was obtained dependent on the species and/or application mode. Antiestrogenic and antiandrogenic compounds resulted also in somewhat contradictory results (Rastogi and Chieffi, 1975). However, experiments were done with different species using diverse exposure regimes, steroidal compounds, and concentrations and thus making generalizations is difficult. Data concerning antiestrogenic or antiandrogenic compounds are scarce, thus there is need to determine whether existing hypotheses about sexual differentiation in amphibians can be verified or should be changed. Adverse effects on reproductive biology are most obvious concerning sexual differentiation and therefore it is important to understand how the basic mechanisms underlying these processes are accomplished.

The main existing hypothesis for sexual differentiation in amphibians deals with primary sex differentiation by genetic sex leading to functioning gonadal sex development during larval stages, which in turn produces sexual steroids responsible for secondary sexual differentiation (Hayes, 1998). In *Xenopus* it is suggested that female genotypes might express preferentially the enzyme aromatase at early larval stages, which in turn converts T into E2 leading to feminization of gonads (Kelley, 1996). Thus the higher endogenous production of E2 by female genotypes would result normally in corresponding female phenotypes such as treatments with exogenous E2 during sensitive larval stages suggesting that E2 might be the main trigger towards sexual differentiation. In addition, the sensitive windows of larval development of *X. laevis* for sex reversal towards feminization by exogenous estrogens were determined by Villalpando and Merchant-Larios (1990). Despite the contradictory findings concerning the potential roles of androgens and antiandrogens (Witschi, 1971; Gallien, 1974; Rastogi and Chieffi, 1975;
Hayes, 1998; Wallace et al., 1999) experiments were neglected to elucidate their impact on sexual differentiation of amphibians and to date basic data of sexual steroid levels and their corresponding receptors during the complete larval development of amphibians were lacking. However, such information including in vivo exposure experiments with estrogenic, antiestrogenic, androgenic, and antiandrogenic compounds are needed for an advanced hypothesis of basic mechanisms of sexual differentiation in amphibians.

In order to obtain more basic information using *X. laevis* as a model, quantification of E2 and androgens during development from fertilized eggs to juveniles by radioimmunoassays (Figs. 1 and 2), and determination of the corresponding mRNA levels of ER and AR in whole body homogenates by semiquantitative RT-PCR (Figs. 3–5) were performed. In addition, short term experiments to demonstrate the genomic impact of exogenous E2 and T on ER- and AR-mRNA expression in vivo were done (Fig. 6) whereas classical exposure experiments should investigate the potential impact on sexual differentiation by the estrogen E2, the antiestrogen tamoxifen, the androgens T, MT, and DHT, and the antiandrogen cyproterone acetate (Fig. 7). An advanced hypothesis on sexual differentiation of *X. laevis*, based on conclusions drawn by results presented here and by recent data of others, is given in Fig. 8.

The first surprising finding was that E2 as well as androgens (sum of T and DHT) were present at high concentrations in eggs, embryos, and hatched larvae. Thus sexual steroids of maternal origin are transferred probably via the normal bioaccumulation into the eggs and accumulated further in developing embryos due to their lipophilic nature because the concentrations of both, E2 and androgens, were higher in freshly hatched tadpoles (stage 38) compared with levels found in complete eggs. E2 as well as androgens decrease dramatically during the first two weeks after hatching when tadpoles reach developmental stages around stage 50. It was obvious that E2 levels became much lower compared with androgens but they showed a tendency to increase already at stage 56, when sexual differentiation of sexes could be determined by gross morphology of gonads, indicating that endogenous production had to be started. From the beginning of secondary
differentiation, the concentrations of E2 were higher in female individuals being significant at stage 62 and much more pronounced in 2-month-old juveniles. Androgens demonstrated decreasing amounts until stage 62, but starting already at stage 56 male individuals contained more androgens than females, which should be due to a different endogenous production in males and females being significant at stages 58 and 66. However, the differences in androgen concentrations between both sexes were not as important as for E2, which is in accordance to measurements of serum levels in adult X. laevis having values for E2 of 3695 ± 895 pg/ml and 187 ± 45 pg/ml and for androgens of 6234 ± 2289 pg/ml and 19 875 ± 3168 pg/ml in females and males, respectively (Kloas, 2002).

Genomic functioning of sexual steroids is coupled to the presence of their corresponding receptors, which was the reason for determining the occurrence of ER and AR indirectly by measuring their mRNAs by semiquantitative RT-PCR. ER-mRNA was determined by using an already existing protocol (Lutz et al., 2002) amplifying a sequence common for all ER-mRNAs detectable in Xenopus (Nishimura et al., 1997). Detection of AR-mRNA in X. laevis (Kelley et al., 1989) by RT-PCR is presented for the first time as a recent methodological development and its applicability is shown in Figs. 4 and 5. The time courses of expression of ER- and AR-mRNA indicated their first presence just after hatching, implicating the concurrent appearance of functional receptors. Both, ER- and AR-mRNA, increased very fast reaching a plateau around stage 50. Therefore, maternal sexual steroids may induce expression of their corresponding receptors, leading to a stage at an early period after hatching that is sensitive for sexual differentiation. This is in agreement with empiric findings about estrogen sensitive responses on sexual differentiation during larval development (Villalpando and Merchant-Larios, 1990). However, some differences exist concerning the time courses of ER- and AR-mRNAs. The elevation of ER-mRNA seemed to be slower compared to that of AR-mRNA and in addition ER-mRNA levels in female individuals were higher indicating significance in 2 months old juveniles. No obvious differences between sexes were found concerning AR-mRNA concentrations suggesting that both mRNAs are regulated at least in part by diverse ways.

In order to get some more information how estrogens and androgens may affect the autoinduction of their corresponding receptors the short term experiment investigating ER- and AR-mRNA expression following a 1-week-exposure to 10^-8 M E2 and T, respectively, was performed using juvenile Xenopus (Fig. 6). E2 treatment caused significant elevations of mRNAs for both, ER and AR, in male and female individuals while T increased mRNA levels only moderately without significance, indicating that T is at least much less efficient compared to E2 suggesting a potential regulatory involvement of other androgens such as DHT.

It is generally accepted that estrogens cause always feminization in sexual differentiation of amphibians (Hayes and Menendez, 1999). Therefore a comparative classical exposure study was performed to determine impacts of all four principle modes of action on sexual differentiation (Fig. 7). Special emphasis was taken for androgens because the use of T and concomitant results in amphibians were discussed contradictory. Our exposure experiments with X. laevis gave results consistent with what is known for mammals (Döhler and New, 1989). Our data showed that estrogens shift sex ratios to feminization as demonstrated before (Kloas et al., 1999), while the antiestrogen tamoxifen led to neutralization caused by underdeveloped gonads keeping a balanced sex ratio. The androgen T did not change sex ratio but MT and DHT induced significant masculinization, while the antiandrogen cyproterone acetate revealed feminization.

One explanation for the findings with androgens is that T is aromatizable and can be further converted into E2, but the two other androgens are not, explaining their efficiency for inducing masculinization via interaction with androgen receptors in responsive target organs. In addition, affinities of T and DHT to AR of Xenopus are similar using a radioreceptor binding assay (unpublished data), but T might also become easier metabolized in target cells in comparison to MT and DHT supporting our findings that exogenous administration of DHT but not of T changed the sex ratios significantly towards male phenotypes. Maternally derived T has to be converted endogenously into DHT by the enzyme 5α-reductase, the preferential expression of which might be the key in genetic males to induce higher levels of DHT leading finally to differentiated testes. The expression of
5α-reductase during development needs to be verified in future experiments.

In contrast, the enzyme aromatase converting T into E2, cannot play an important role for female gonadal development. Marked aromatase expression was found only at the end of larval development, and in vivo treatment with aromatase inhibitors did not change significantly the sex ratio (Miyata et al., 1999; Miyashita et al., 2000). In vivo the initial presence of low aromatase expression at stage 51 has been documented (Miyata et al., 1999), which seems to be too late to affect significantly sexual differentiation by endogenous conversion of T, derived from exogenous or endogenous sources, into E2. Therefore indirect evidence is obtained that a pronounced activity of 5α-reductase preferentially expressed in genetic males might be the natural trigger for induction of sexual differentiation in Xenopus towards masculinization. The involvement of aromatase by endogenous production of E2 for feminization seems to be negligible under normal in vivo conditions because a complete sex reversal triggered by E2 treatment in vivo could only be obtained until stage 50 during larval development of X. laevis (Villalpando and Merchant-Larios, 1990). It seems to be questionable whether the start of aromatase-mRNA expression at stage 51 may immediately lead to marked endogenous E2 production and, in addition, there is need to prove that aromatase is regulated differently by both sexes. However, any endogenous production of E2 would be too late to affect normal sexual differentiation of genetic females into female phenotypes, which suggests the other way around that in general differentiation is directed towards feminization and needs only a special trigger for masculinization.

4.1. Recent hypothesis for sexual differentiation in Xenopus laevis

In X. laevis the genotype of males is homogametic ZZ, whereas females resemble the heterogametic ZW genotype, but it is impossible to differentiate the two genotypes because of the absence of heteromorphic sex chromosomes (Schmid and Steinlein, 1991). Under normal conditions tadpoles develop into 50% males and 50% females according to their genetic sex, however, the sexual steroids derived from maternal origin are evenly distributed. The discussion about endogenous production of sexual steroids is still a matter of debate (Kelley, 1996; Hayes, 1998), because of the lack of experiments demonstrating clearly functioning synthesis of sexual steroids during larval development. It seems to be very likely, based on our recent findings indicating an increase of E2 and androgens levels during metamorphosis, that sexual steroids might be synthesized endogenously during late developmental stages by the already differentiated gonads suggesting that endogenous production of sexual steroids is not needed for sexual differentiation because it starts after the sexual phenotype is accomplished.

Sexual differentiation can be shifted by modulation of estrogen and androgen receptors or by supplements of estrogens and androgens, and thus the real response is dependent on the complex interplay of number of functional receptors and the relation between the natural androgen DHT and E2. Since E2 and androgens (T and DHT) of maternal origin are equally distributed in all eggs, changes in the relation between DHT and E2 are caused by converting the maternal source of T into DHT by 5α-reductase. We suggest that genetic females have negligible 5α-reductase activity, resulting in a low DHT/E2 ratio. In genetic males, the enzyme activity might be higher, leading to a high DHT/E2 ratio. The differences in DHT/E2 ratios cause feminizing and masculinizing gonads, respectively.

Addition of exogenous administration of estrogens and androgens may thus also affect sexual differentiation by shifting the relation between DHT and E2 like compounds. In contrast antiandrogens suppress the androgen receptor mediated cellular processes, leading indirectly to a lower functioning DHT/E2 ratio resulting in feminization. Antiestrogens block all estrogen induced developmental processes in genetic females and males resulting in a general depression of gonadal development leading to neutralization. It is probable that expression of estrogen and androgen receptors could be down regulated by antiestrogen, and thus antiestrogen is unlikely to have effects on the sex ratio rather than causing neutralization of gonads by deteriorated development.

Similar investigations using other anuran species are needed to prove whether the hypothesis presented here may serve as a general model for sexual differentiation in anurans. Data on sexual differentiation in urodeles are scarce and lack experiments using their naturally occurring androgens, DHT and 11-ketotestosterone, and antiandro-
Androgenic modes of action in urodeles may differ markedly from these in anurans because urodeles possess the androgens T, DHT, and 11-ketotestosterone. The latter is lacking in anurans and thus urodeles more closely resemble teleosts than anurans concerning androgens (Moore, 1987). Sex reversal in urodeles can be accomplished, as in anurans, by estrogens leading to feminization but addition of the androgen T demonstrated negligible or unexpected feminizing effects (Wallace et al., 1999) suggesting that the masculinizing functioning androgens should be DHT or 11-ketotestosterone. Thus it is obvious that urodeles may represent a specific amphibian type regarding their sexual differentiation (Clark et al., 1998) particularly masculinization. This might be due to distinct androgens, and maybe also to differences in the specificity of their androgen receptors in comparison to anurans. However, in principle it is possible that our hypothesis presented here for *Xenopus* could also be transferred as mode of action for sexual differentiation in urodeles, which required additional investigations in urodeles to elucidate the basic mechanisms of the responsible processes.

Future experiments on sexual differentiation are needed to verify or falsify our recent hypothesis for sexual differentiation. The determination of basic mechanisms of sexual differentiation may provide information being the prerequisite for risk assessment of EDC concerning reproductive biology. In order to get more detailed information about endogenous production of sexual steroids experiments for determining tissue and stage dependent regulation of aromatase and 5α-reductase are required and should include measurements of both androgens, T and DHT, separately, which could not be done with our present available assay measuring a sum of T and DHT because the antibody used has same affinity for T such as for DHT. The specific interplay between sexual steroids and corresponding ER and AR must be studied more in detail. Our present work just summarizes the whole body contents of mRNAs for ER and AR. However, it is very probable that tissue specific differences concerning ER and AR expression can be found during larval development especially between both sexes. In addition, it might be probably found that severe classes of ER and AR exist also in amphibian species such as in all other vertebrate classes, which would make the whole picture even more complicated. Establish-