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RESEARCH ARTICLE

# Effect of methyl testosterone- and ethynyl estradiol-induced sex differentiation on catfish, *Clarias gariepinus*: expression profiles of *DMRT1*, Cytochrome P450aromatases and 3 β-hydroxysteroid dehydrogenase

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**Abstract** The objective of the present study is to observe the effect of exogenous steroids, methyl testosterone (MT) and ethynyl estradiol (EEL) on gonadal differentiation and analyze its effect on the expression of several genes during testicular and ovarian differentiation in juvenile catfish. Exogenous hormone treatments (MT and EEL) were given by immersion at different days of hatching. The histological analysis revealed that the EEL- and MT-treatments resulted in the initiation of ovarian and testicular differentiation, respectively. This is further supported by specific expression of two forms of *DMRT1* in the MT-treated group but not in the EEL-treated group at 47 days after hatching (dah). The reverse is true for the expression of ovarian aromatase. Results of the semi-quantitative RT-PCR show that brain aromatase transcript levels are high in 47 dah control (histologically female) and 47 dah EEL-treated fish, as compared to 47 dah MT-treated fish. At 60 dah, brain aromatase showed elevation in its expression. Interestingly, the expression pattern of 3 β-HSD did not show any change in EEL- and MT-treated fish. The present study also provides a strategy to study sex differentiation, for those species where genetic sex population is unavailable.

**Keywords** 3 β-HSD · Cytochrome P450 aromatases · *DMRT1* · Ethynyl estradiol · Methyl testosterone

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## Introduction

Sex differentiation in the fish show high plasticity as it can be influenced by several factors like genetic sex, temperature and exogenous steroid hormones (Nagahama et al. 2004). Genetic sex can be reversed functionally by exposure to androgens or estrogens during critical period of sex differentiation or at juvenile stage (Piferrer et al. 1993; Nagahama et al. 2004). Monosex population can be obtained by crossing experimentally produced YY or XX males

with normal females (Scholz et al. 2003). This approach has been widely used in fishes like the Nile tilapia, medaka, zebrafish and rainbow trout in order to study the expression of several genes during gonadal and brain sex differentiation (Scholz et al. 2003; Matsuda et al. 2002; Sudhakumari et al. 2003). Such an approach was not attempted in subtropical annual breeders like catfish. To study gonadal sex differentiation, it is essential to track down the expression of sex-specific genes. *DMRT1* is expressed male specifically in testis during sex differentiation, suggesting its importance in testicular differentiation (Nagahama et al. 2004). Though two forms of cytochrome P450aromatase (ovarian [oP450arom] and brain [bP450arom] forms) have been identified in tilapia and other species, oP450arom seems to play a major role in ovarian differentiation (Sudhakumari et al. 2003). More recently, in addition to oP450arom, Foxl2 is found to be more critical gene to impart ovarian differentiation and function (Wang et al. 2004). The gonadal differentiation period is different in each species based on its breeding and developmental pattern. These variations create immense diversity on the ontogeny of various sex specific genes. In the present study, we aimed to study the expression pattern of sex-specific genes such as *DMRT1* and oP450arom after driving catfish fries to testicular and ovarian differentiation by treating them with exogenous steroids. An attempt was made to understand brain sex differentiation by studying the expression of bP450arom. We also intend to analyze the expression of 3 $\beta$HSD to monitor steroidogenic activity, as it is one of the markers for the initialization of steroidogenesis. To enable this study, we have cloned species-specific partial cDNA fragments of *DMRT1*, aromatases and 3 $\beta$HSD from catfish, *Clarias gariepinus*.

## Materials and methods

Adult catfish *C. gariepinus* bred in our laboratory were used for the extraction of total RNA from gonads and brain using TRI reagent (Sigma) according to the manufacturer's protocol. First strand cDNA was synthesized from 5 $\mu$g of total RNA with oligo-(dT) 12–18 primer using Superscript-III (Invitrogen) according to manufacture's instructions. Degenerate primers designed from other vertebrate aromatases (forward primer [frd]: 5′TGG WYK GGN ATH GGB ACG GC3′, reverse primer[rev]: 5′GGV CCD GTB ARV GCT TTR G3′), 3 $\beta$HSDs (frd: 5′GTS AAY STS AAA GGW ACC MA3′, rev: 5′GGN GTG TCR TCK GAG AKR WAR TA3′) and specific primers for *DMRT1*, designed from conserved domain of vertebrate DMRT1s (frd1: 5′ATG CGT GGC TGT TTT TTA CCC ACC3′, frd2: 5′ATG CCG AAG TGC TCC CGG TGC AGG3′ and rev: 5′TAG TAG GAG TGC ATA CGG TAC3′), were used to clone respective partial cDNA fragments. All the cDNA fragments obtained were cloned into pGEM-T Easy (Promega) vector before sequencing. All the clones were bidirectionally determined in ABI prism 316 sequencer.

Catfish fries were obtained by *in vitro* fertilization. Treatments of methyl testosterone (MT) and ethynyl estradiol (EEL) with a concentration of 500 $\mu$g/l was started from the day of hatching and were given 5 additional times at 4, 8, 12, 16 and 21 days after hatching (dah). The duration of treatment on day one is for 3 h in the case of MT and 1.5 h for EEL. Rest of the treatments was done at each time for 3 h. A control group was maintained. Eighty fries were used in each group, where 10% mortality rate in MT-treated group and 30% mortality rate in EEL-treated group was observed during treatment. Expression of DMRT1, 3 $\beta$HSD, and oP450arom at 47 and/or 60 dah by RT-PCR and for bP450arom semi-quantitative RT-PCR in MT, EEL and control fish was carried out by following the method of Kwon et al. (2001). Five fishes from each group at different dah were used for RT-PCR and histological analysis. Histological analysis (hematoxylin and eosin staining) of gonad of MT- and EEL-treated fish and control fish at 47 and 75 dah was also done.

## Results

Based on preliminary histological analysis, sex ratio (male:female) for control group was 1:9, for MT-treated group it was 10:0 and for EEL-treated group it was 0:10. The PCR amplification made by *DMRT1* (Fig. 1a and b) specific primers gave two products of 421 and 524 base pairs (bp), which showed complete homology to the previously cloned *C. gariepinus*

**Fig. 1** Partial amino acid sequences of (**a**) *Dmrt1* isoform 1, (**b**) *Dmrt1* isoform 2, (**c**) bP450arom and (**d**) 3 $\beta$-HSD

**(a)** *Dmrt1* **isoform 1**
MRGCFLPTGPGDRGVATSRNGFLFCSSSLISARPRASAASGSRSSLTPSPTAATRGHS
EGSADLVVDASYYNFYQPSRYPAYYSNLYNYQQYQQMPSGDSRLSSHNMSQQYRM
HSY

**(b)** *Dmrt1* **isoform 2**
MPKCSRCRNHGFVSPLKGHKRLCNWRDCQCQKCKLIAERQRVMAAQVALRRQQ
AQEEEMGICTPVNLSGSDIVVKDEPGNDYGFAVGARSLASSPAASGSRSSLTPSPTAA
TRGHSEGSADLVVDASYYNFYQPSRYPAYYSNLYNYQQYQQMPSGDSRLSSHNMSQ
QYRMHSY

**(c)** **bP450arom**
GLGIGTASNYYNKKYGCMTRVWIQGEETLILSKSSAVYHVLKSSNYVARFASRSGL
RCIGMDEQGIIFNSNIPLWKKLRTYFAKALTG

**(d)** **3β-HSD**
VNVKGXXLLLEACIQENVASFIYTSSIEVAGPNHRGDPVINGHEDTVYYSYLKFSYSQ
TKKEAEQLCLSAQGEILPNGGRLATCALRPMYIYGEGCRFTLGHMRDGIQNGDVL
LRNSRHDAKVNPVYVGNVTLAHLQAARALREPQTRAVVGGNFYFLSD

*DMRT1* (one form). The cDNA fragments of bP450arom (225 bp, Fig. 1c), and 3 $\beta$-HSD (489 bp, Fig. 1d) showed high homology to respective cDNAs of channel catfish. The details and homology of the RT-PCR performed using degenerate primers for amplifying oP450arom (267 bp) was mentioned earlier (Rasheeda et al. in the present volume). The histological analysis revealed the initiation of differentiation of ovary and testis in the EEL- and MT-treated groups, respectively. This is further supported by the specific expression of two forms of *DMRT1* in the MT-treated group but not in EEL-treated group at 47 dah (Fig. 2a and b). The oP450arom expression was evident in the EEL-treated group but not in MT-treated group (Fig. 2c). The expression pattern of 3 $\beta$-HSD did not show any change in EEL- or MT-treated group (Fig. 2d). Semi-quantitative RT-PCR of bP450arom showed increased expression in the EEL-treated and control groups (histologically differentiating female) as compared to MT-treated group at 47 dah in brain (Fig. 3a). Expression of bP450arom in brain of the MT-treated showed slight elevation at 60 dah (Fig. 3b). Histological analysis at 47 and 75 dah of the MT- and EEL-treated group (Figs. 4, 5) showed signs of initiation of testicular and ovarian differentiation, respectively. On the other hand, control group at 75 dah showed indication of ovarian (Fig. 5c) and testicular differentiation in different fish samples (Fig. 5d and e).

## Discussion

Present study was aimed at using *DMRT1* and oP450arom as a marker to understand testicular and ovarian differentiation in catfish where genetic XX and XY population is unavailable. Though full length *DMRT1* is already cloned in this species identification of two altered forms of *DMRT1* gains importance to implicate its role in testicular development. However, in the present study, we are not sure whether the MT- or EEL-treatment had

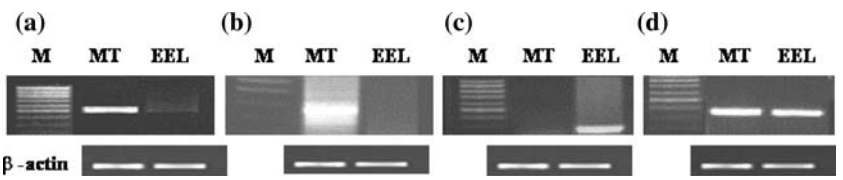


**Fig. 2** RT-PCR analysis (representative gel, $n$=5) in gonads of MT- and EEL-treated fish at 47 dah (**a**) Expression of *DMRT1* isoform 1 [421 bp], (**b**) Expression of *DMRT1* isoform 2 [524 bp], (**c**) Expression of oP450arom, (**d**) Expression of 3 $\beta$HSD

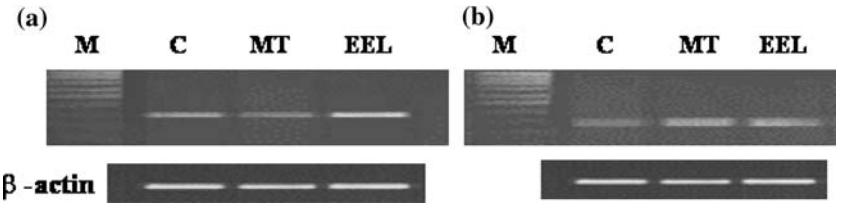


**Fig. 3** Semi-quantitative RT-PCR analysis (representative gel, $n = 5$) in brain of MT- and EEL-treated fish at 47 and 60 dah (**a**) Expression of bP450arom at 47 dah, (**b**) Expression of bP450arom at 60 dah

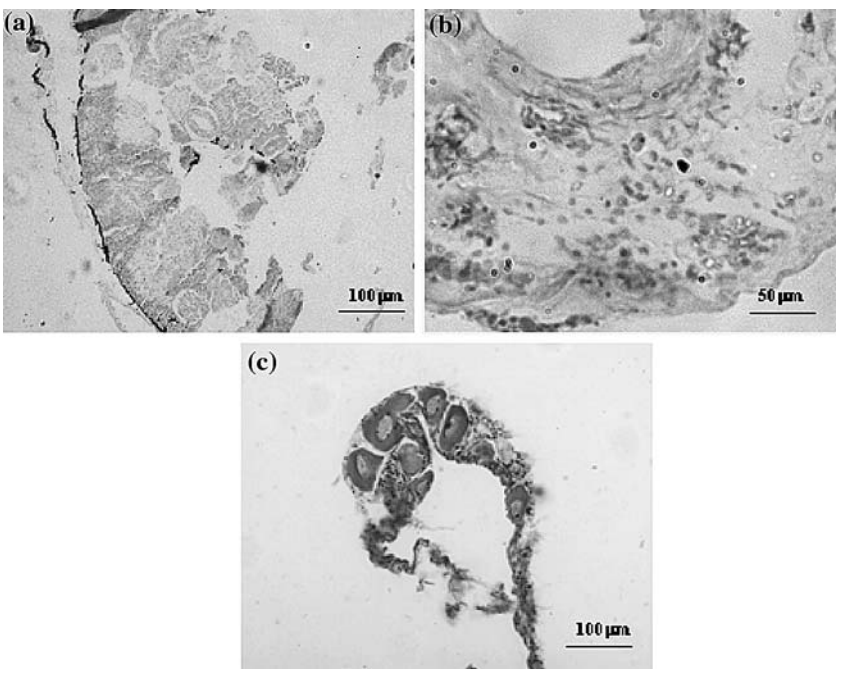


**Fig. 4** Histology of control, MT- and EEL-treated fishes at 47 dah. (**a**) Gonad of EEL-treated fish, (**b**) Gonad of MT-treated fish, (**c**) Gonad of control fish

influenced sex reversal, as we did not use genetic sex populations. Nevertheless, presence of *DMRT1* in MT-treated group indicated that MT-treatment is effective to initiate testicular development. It is also possible that the treatment of MT and EEL was given well before the critical period of sex differentiation, which might have decided the course of testicular or ovarian differentiation. Based on our preliminary observation, the initiation of gonadal differentiation starts around 40–50 dah and complete differentiation (ovary) was evident at 75 dah in the catfish. It seems that testicular differentiation requires more time than ovarian differentiation in catfish. More in depth analysis are required for precise identification of gonadal differentiation. It is also necessary to observe the changes in gonadal differentiation till 90–100 dah. Absence of *DMRT1* in EEL-treated group further supported our observations. On the other hand, presence of the expression of oP450arom confirms initiation of ovarian differentiation in the EEL-treated group. The EEL-treated fish also showed delayed ovarian development when compared to normal ovarian development. This gives a clue that driving ovarian differentiation using EEL might be slower than driving testicular differentiation using MT. MT- and EEL-treatment showed minor changes in the expression pattern of bP450arom in catfish brain, which was not observed in the Nile tilapia (Sudhakumari et al. 2003). This might be due to non-usage of genetic sex population in the present study. No change in the expression pattern of 3

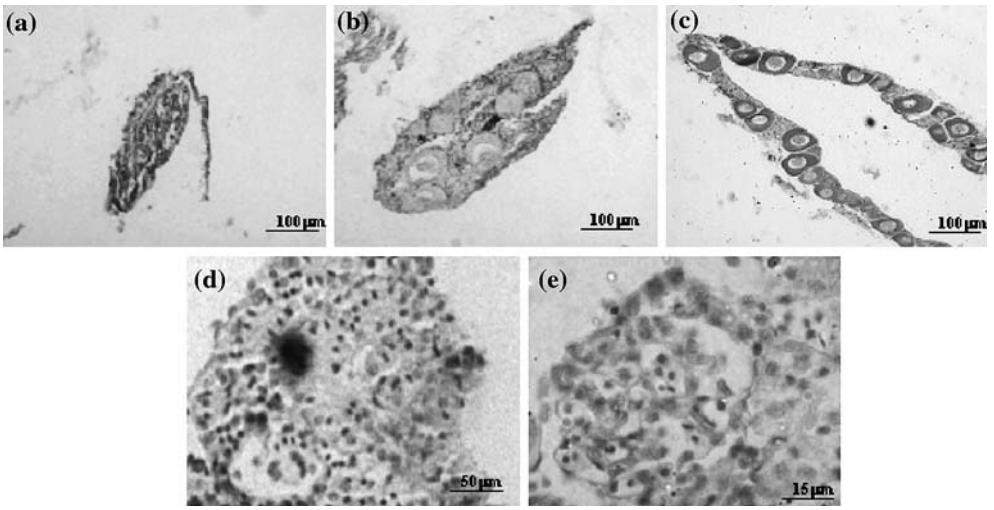


**Fig. 5** Histology of control, MT- and EEL-treated fishes at 75 dah. (**a**) Gonad of MT-treated fish, (**b**) Gonad of EEL-treated fish, (**c**) Gonad of control fish (differentiated female), (**d**) Gonad of control fish (differentiated male), (**e**) Gonad of control fish (differentiated male; in high magnification)

$\beta$-HSD between MT- and EEL-treated fish indicated that the $\Delta 4$ steroids such as progesterone are not altered.

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