

Brief Exposure of Embryos to Steroids or Aromatase Inhibitor Induces Sex Reversal in Nile Tilapia (*Oreochromis niloticus*)



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ABSTRACT

This study aimed to develop sex reversal procedures targeting the embryonic period as tools to study the early steps of sex differentiation in Nile tilapia with XX, XY, and YY sexual genotypes. XX eggs were exposed to masculinizing treatments with androgens (17 α -methyltestosterone, 11-ketotestosterone) or aromatase inhibitor (Fadrozole), whereas XY and YY eggs were subjected to feminizing treatments with estrogen analog (17 α -ethynylestradiol). All treatments consisted of a single or double 4-hr immersion applied between 1 and 36 hour post-fertilization (hpf). Concentrations of active substances were 1000 or 2000 $\mu\text{g l}^{-1}$ in XX and XY, and 2000 or 6500 $\mu\text{g l}^{-1}$ in YY. Masculinizing treatments of XX embryos achieved a maximal sex reversal rate of 10% with an exposure at 24 hpf to 1000 $\mu\text{g l}^{-1}$ of 11-ketotestosterone or to 2000 $\mu\text{g l}^{-1}$ of Fadrozole. Feminization of XY embryos was more efficient and induced up to 91% sex reversal with an exposure to 2000 $\mu\text{g l}^{-1}$ of 17 α -ethynylestradiol. Interestingly, similar treatments failed to reverse YY fish to females, suggesting either that a sex determinant linked to the Y chromosome prevents the female pathway when present in two copies, or that a gene present on the X chromosome is needed for the development of a female phenotype. *J. Exp. Zool.* 323A:31–38, 2015. © 2014 Wiley Periodicals, Inc.

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Sex reversal techniques are largely used for the control of sex in fish farming and in fundamental studies on sex determinism mechanisms. In tilapia aquaculture, all-male populations are preferred because they achieve higher growth rate and prevent uncontrolled reproduction (Beardmore et al., 2001). Due to its efficiency and simplicity, one of the most common practices used to produce mono-sex populations of tilapia or to obtain males and females with particular sexual genotypes is the sex reversal through the dietary administration of sex steroids during the sensitive period of sex differentiation, usually from 10 to 38 days post-fertilization (dpf) (Phelps and Popma, 2000). Sex reversal treatments are also useful to explore the role played by genetic

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and endocrine factors involved in the mechanisms of sex determinism and differentiation.

In fish, a major sex-determining gene similar to the mammalian *Sry* was identified in a few species, but not in the Nile tilapia *Oreochromis niloticus* (Kikuchi and Hamaguchi, 2013). In this species, a sex-specific expression of genes potentially involved in sexual differentiation was reported in the gonad (*foxl2*, forkhead transcription factor L2; *cy19a1a*, cytochrome P450, family 19, subfamily A, gonad aromatase; *dmrt1*, doublesex and mab-3-related transcription factor 1) (Ijiri et al., 2008) and in the brain (*amh*, anti-Müllerian hormone) (Poonlaphdecha et al., 2011) from 9–10 dpf but not deeply investigated earlier during the embryonic and larval development. Classically, sex reversal treatments override the natural process of sex differentiation by acting after 10 dpf on mechanisms taking place during the gonad morphogenesis. The masculinizing hormone 17 α -methyltestosterone (MT) was reported to up-regulate *dmrt1*, a gene involved in testicular differentiation, from 19 dpf in XX tilapia (Kobayashi et al., 2008). Similarly, administration of 17 α -ethynylestradiol (EE2) to XY fry induced the ovarian differentiating pathway by repressing *dmrt1* and promoting the expression of *cyp19a1a* at 19 dpf (Kobayashi et al., 2003, 2008).

However, an increasing body of evidence gathered in different species argue for a precocious initiation of the differentiation pathway that could occur in the brain or in the primordial germ cells before the development of the gonad (Tsai et al., 2000; D’Cotta et al., 2001; Kobayashi and Iwamatsu, 2005; Kobayashi et al., 2008; Rougeot et al., 2008a,b; Blázquez and Somoza, 2010). Therefore, it is particularly interesting to target sex reversal treatments on early developmental stages in order to study the upstream mechanisms and trace the sex determinism cascade up to the major determinants. During ontogenesis, the brain starts to morphologically differentiate from 31 hour post-fertilization (hpf) and the primordial germ cells can be identified from 46 hpf in Nile tilapia (Morrison et al., 2001). Then, primordial germ cells migrate to the genital ridge to form a gonadal primordium by the 7th dpf (Kobayashi et al., 2003). Based on the assumption that the first steps of molecular sex differentiation would occur during these precocious developmental events, it could be possible to modify the sex determinism by treatments applied during the embryonic period.

In medaka, *Oryzias latipes*, successful feminizing and masculinizing sex inversions were performed by brief exposure of freshly fertilized eggs to 17 β -estradiol for 24 hr (Iwamatsu et al., 2005; Kobayashi and Iwamatsu, 2005), and preovulatory oocytes to 17 α -methyl dihydrotestosterone for 10 hr (Iwamatsu et al., 2006), respectively. Unlike tilapia, the major sex-determinant (*dmy*) was identified in medaka (Matsuda et al., 2002), but interestingly these two species have similar sex determinism systems and morphogenesis during sex differentiation (Siegfried, 2010), suggesting that such precocious

treatments could be efficiently applied in tilapia. In Mozambique tilapia (*Oreochromis mossambicus*), Rosenstein and Hulata, ('92) tested various treatment schemes with a double immersion (of 2–28 hr) of eggs at 2 and 4 dpf in 17 β -estradiol solutions (200–1500 $\mu\text{g l}^{-1}$) but did not observe any feminizing effect. Using more prolonged treatments (from fertilization to hatching) with EE2 (500 $\mu\text{g l}^{-1}$) and MT (1000 $\mu\text{g l}^{-1}$) in *O. niloticus*, Rougeot et al. (2008a) obtained up to 79% of feminization and 20% of masculinization. However, this procedure is not the most appropriate to study the embryonic developmental events as the prolonged exposure led to an important accumulation of hormones that could act by a delayed effect on the process of gonad differentiation in fry. Characterization of the EE2 clearance profile in XY fish submitted to such a feminizing treatment revealed very high extra-physiological concentrations (more than 5000 ng g^{-1}) of EE2 in fry at the onset of the period of gonadal differentiation (10 dpf) (unpublished data).

The objective of the present research was to develop sex reversal procedures in Nile tilapia using brief immersion treatments applied on the first stages of development in order to focus the action of hormones only on embryogenesis. Different masculinizing and feminizing schemes, varying in active substances, concentrations and moments of application, were tested on XX, XY, and YY spawns, respectively, to identify the most efficient procedures that might be used to study the early mechanisms of sex differentiation.

MATERIALS AND METHODS

Reproduction and Egg Incubation

Nile tilapia *O. niloticus* (Lake Manzala strain) originated from the Research and Education Center in Aquaculture (CEFRA), University of Liège (Belgium). Different full-sib families were produced by artificial reproduction (Gennotte et al., 2012). XX spawns were obtained by crossing XX males with XX females, XY spawns by crossing YY males with XX females, and YY spawns by crossing YY males with YY females. Broodstock fish were individually stocked in 125-l aquaria at 27 °C with a 14 hr light/10 hr darkness photoperiodic regime. Fish were fed at satiation with special broodstock commercial tilapia diet (45% proteins, 5% lipids, Coppens—The Netherlands). After artificial fertilization, eggs were counted, distributed in different batches according to treatments and incubated in 1.5l Zug bottle at 27 °C. Fertilization rate was evaluated on 100 eggs after the first mitotic cleavage (2 hpf) (Morrison et al., 2001).

Experiments were carried out according to the guidelines of the University of Liège ethical committee and the European animal welfare recommendations.

Hormone and Aromatase Inhibitor Solutions

Steroid hormones 17 α -ethynylestradiol (EE2), 17 α -methyltestosterone (MT), 11-ketotestosterone (11KT), and the aromatase

inhibitor Fadrozole (Fa) were purchased from Sigma–Aldrich. Hormones and Fa were dissolved in 100% ethanol to prepare 1000-fold concentrated stock solutions and stored at 4 °C. For immersion treatments, 1 mL of stock solution was added to 1 l of hatchery water. Control groups were incubated in 1:1000 ethanol.

Hormonal Treatments

Feminizing and masculinizing treatments consisted of a single or a double immersion in hormonal or Fa solutions. All immersions lasted 4 hr. Treatments were performed at different stages of development from 1 to 36 hpf. Eggs were transferred from the Zug bottle into a 1 l glass beaker filled with hatchery water and maintained in a thermostatic bath at 27 °C. One milliliter of stock solution (ethanol for control groups) was added to the water. During incubation, water was oxygenated by an air diffuser. After 4 hr of immersion, eggs were netted, gently soaked up and rinsed in six different baths to remove hormone residues. The first three baths contained 1:1000 ethanol and the next three only hatchery water. Control groups were handled in the same way. After rinsing, eggs were replaced into the hatchery.

A total of 10 different masculinizing treatments were tested on XX spawns, and 6 and 5 feminizing treatments on XY and YY spawns, respectively. XX eggs were treated with MT (1000 and 2000 $\mu\text{g l}^{-1}$), an aromatizable synthetic androgen, 11KT (1000 and 2000 $\mu\text{g l}^{-1}$), a non-aromatizable natural androgen, and Fa (1000 and 2000 $\mu\text{g l}^{-1}$), a non-steroidal inhibitor of aromatase, the enzyme that converts androgens into estrogens. XY and YY eggs were treated with EE2 (2000 and 6500 $\mu\text{g l}^{-1}$), a synthetic estrogen.

The concentrations of hormones used in XX and XY treatments were based on the literature (Gilling et al., '96; Kobayashi et al., 2003; Wassermann and Afonso, 2003) and on our previous experiments of egg immersion (Rougeot et al., 2008a). In YY treatments a first concentration of EE2 equal to the XY treatments was tested, and a second concentration increased in the same proportion as EE2 concentrations used in dietary hormonal treatments (150 mg kg^{-1} for XY and 500 mg kg^{-1} for YY) in our laboratory (unpublished data). As a differential timing of sex differentiation can be suspected in YY compared to XY (Herrera and Cruz, 2001), different moments of treatment application were tested in YY. Hormone and Fa concentrations, and the moment of application of each treatment figure in the result tables (Tables 1–3).

Each spawn was divided into several batches receiving different treatments and a control group for each moment of treatment application. In YY groups immersed in a 6500 $\mu\text{g l}^{-1}$ EE2 solution at different moments, only one control group immersed at 24 hpf was used. The number of fertilized eggs per batch and the number of spawns per treatment ranged from 78 to 1371 and from 2 to 8, respectively.

MT and EE2 are routinely used in our laboratory for the feminization of XX and the masculinization of XY and YY fry,

respectively by dietary administration (from 10 to 40 dpf, XX: 65 mg MT kg^{-1} food; XY: 150 mg EE2 kg^{-1} food; YY: 500 mg EE2 kg^{-1} food). These treatments succeed in 94–100% of sex reversal (unpublished data).

Juvenile Rearing and Sex-Ratio Analysis

At 8 dpf, larvae were counted in order to calculate the survival rate ($= 100 \times n \text{ larvae} / n \text{ fertilized eggs}$) and transferred into 50-l aquaria (27 °C). From 10 dpf, fish were fed close to satiation six times a day with a commercial tilapia diet (47% proteins, 8% lipids, Coppens–The Netherlands). Phenotypic sex was determined by the acetocarmine squash method (Guerrero and Shelton, '74) at 90 dpf. Fish were euthanized by overdose (500 mg l^{-1}) of benzocaine (Sigma–Aldrich) and a piece of gonad was microscopically examined after acetocarmine coloration. The number of sexed fish per batch ranged from 22 to 121 (median = 74), from 8 to 124 (median = 50) and from 7 to 101 (median = 69) for XX, XY, and YY, respectively. Adding together the different spawns, the overall number of sexed fish per treatment ranged from 69 to 553.

Data Analysis

Survival (at 8 dpf) and sex reversal rates are expressed as the overall value and the total range (minimum and maximum) of all the spawns exposed to the same treatment. Sex ratios and survival of the treated groups were compared against the values of their corresponding control groups using the 2×2 contingency chi-square (χ^2) test ($df = 1$). Values were considered as significant at $P < 0.05$.

RESULTS

XX Masculinization Experiments (Table 1)

At 8 dpf, survival rates in XX spawns ranged from 22 to 86% and from 25 to 64% in treated and control groups, respectively. Overall survival values were significantly higher in batches exposed to MT in a single immersion (at 1 or 24 hpf) (from 48 to 61%) than in their respective controls (39–43%). In fish submitted to a double immersion (at 1 and 24 hpf), survival was similar in control and treated batches (from 35 to 42%). In 11KT and Fa treatments, overall survival was 59% in controls and ranged from 51 to 70% in treated groups with significantly higher values in fish exposed to 1000 $\mu\text{g l}^{-1}$ of 11KT and Fa and a lower value in fish exposed to 2000 $\mu\text{g l}^{-1}$ 11KT l^{-1} .

All the XX control fish were females as expected. None of the MT treatments induced a significant deviation of the sex-ratio. In one spawn, immersion in 1000 $\mu\text{g l}^{-1}$ of MT at 1, 24, and 1 + 24 hpf, respectively induced 8.1, 8.3, and 9.1% of males but the numbers of sexed fish were low in these batches ($n < 37$) and deviations were not significant. On the contrary, treatments with 11KT or Fa significantly induced up to 10% of sex reversal. Overall percentages of males in 11KT and Fa treatments were

Table 1. Effect of androgen and aromatase inhibitor treatments applied in a single or double 4 hr-immersion at different moments of the embryonic development (1 and/or 24 hr post-fertilization, hpf) on the survival (at 8 days post-fertilization, dpf) and the sex ratio of XX Nile tilapia fry (11KT: 11-ketotestosterone; Fa: fadrozole; MT: 17 α -methyltestosterone; * $P < 0.05$).

| Active substance | Concentration ($\mu\text{g l}^{-1}$) | Moment (hpf) | n XX spawns | % Survival (8 dpf) | | | | Total n sexed | % Sex reversal | | | | | |
|------------------|----------------------------------------|--------------|-------------|--------------------|-----|---------|----------|---------------|----------------|------|------|---------|----------|---|
| | | | | Min | Max | Overall | χ^2 | | P | Min | Max | Overall | χ^2 | P |
| Control | 0 | 1 | 3 | 25 | 62 | 39 | | 123 | 0.0 | 0.0 | 0.0 | | | |
| MT | 1000 | 1 | 3 | 29 | 74 | 48* | 5.9 | 173 | 0.0 | 8.1 | 1.7 | 2.2 | 0.142 | |
| MT | 2000 | 1 | 3 | 23 | 86 | 60* | 31.5 | 211 | 0.0 | 0.0 | 0.0 | 0.0 | >0.999 | |
| Control | 0 | 24 | 3 | 32 | 56 | 43 | | 133 | 0.0 | 0.0 | 0.0 | | | |
| MT | 1000 | 24 | 3 | 22 | 82 | 53* | 6.5 | 194 | 0.0 | 8.3 | 1.0 | 1.4 | 0.240 | |
| MT | 2000 | 24 | 3 | 49 | 74 | 61* | 24.6 | 220 | 0.1 | 2.6 | 0.9 | 1.2 | 0.270 | |
| Control | 0 | 1+24 | 2 | 29 | 46 | 36 | | 90 | 0.0 | 0.0 | 0.0 | | | |
| MT | 1000 | 1+24 | 3 | 25 | 61 | 42 | 1.5 | 145 | 0.0 | 9.1 | 3.5 | 3.2 | 0.075 | |
| MT | 2000 | 1+24 | 2 | 35 | 35 | 35 | 0.3 | 90 | 0.0 | 2.6 | 1.1 | 1.0 | 0.316 | |
| Control | 0 | 24 | 6 | 37 | 64 | 59 | | 453 | 0.0 | 0.0 | 0.0 | | | |
| 11KT | 1000 | 24 | 3 | 27 | 59 | 63* | 4.4 | 150 | 0.0 | 10.0 | 3.3* | 6.8 | 0.009 | |
| 11KT | 2000 | 24 | 3 | 25 | 63 | 51* | 10.2 | 200 | 0.0 | 8.0 | 3.5* | 7.1 | 0.008 | |
| Fa | 1000 | 24 | 3 | 50 | 77 | 70* | 22.0 | 250 | 1.0 | 7.0 | 4.4* | 9.0 | 0.003 | |
| Fa | 2000 | 24 | 6 | 44 | 71 | 58 | 0.0 | 464 | 0.0 | 10.0 | 3.9* | 17.9 | <0.001 | |

similar in 1000 and 2000 $\mu\text{g l}^{-1}$ immersions (3.3 and 3.5%, 4.4 and 3.9% for 11KT and Fa, respectively) and significantly different from the control groups.

XY Feminization Experiments (Table 2)

Survival rates in XY spawns ranged from 26 to 85% and from 28 to 86% in EE2-treated and control groups, respectively. Overall survival values were above 60% in groups treated with a single immersion at 1 or 24 hpf and not statistically different from the control groups, except for the group treated in a 1000 $\mu\text{g l}^{-1}$ solution at 1 hpf that displayed a higher survival (72%). Double immersion at 1 and 24 hpf led to lower survival. In this treatment, survival of hormone-exposed fish (32–33%) was significantly lower than the controls (44%).

Sex-ratio of the XY control groups was 100% male as expected. A significant sex reversal effect ($P < 0.001$) was observed in all the EE2 treatments. Fish exposed to EE2 at 1, 24, and 1+24 hpf showed increasing overall sex reversal rates: 9.9–26.6% at 1 hpf, 48.0–48.6% at 24 hpf, and 65.9–65.2% at 1+24 hpf for 1000 and 2000 $\mu\text{g l}^{-1}$, respectively. The efficiency of the treatment was related to the hormone concentration only in the treatment applied at 1 hpf. The maximum sex reversal rate (91%) was observed in a spawn immersed at 24 hpf in a 2000 $\mu\text{g l}^{-1}$ solution.

YY Feminization Experiments (Table 3)

In treatment of YY at 6500 $\mu\text{g EE2 l}^{-1}$, overall survival was greatly affected in fish immersed at 12 hpf (24% vs. 64% in the controls). For the treatment applied later (18, 24, and 36 hpf), survival was similar to the controls. Survival values are missing for the treatment at 2000 $\mu\text{g l}^{-1}$.

All the YY control fish were males as expected. None of the EE2 treatment significantly skewed sex-ratio in any of the spawns.

DISCUSSION

Our results demonstrate that brief 4 hr-immersions of fertilized eggs in androgen, aromatase inhibitor, and in estrogen solutions applied between 1 and 24 hpf can induce significant sex reversal in XX and XY Nile tilapias. Feminization of XY with EE2 is 10 to 15-fold more efficient than masculinization of XX with 11KT or Fa. No significant masculinizing effect was observed with MT. Interestingly, estrogen treatments failed to reverse the sex of YY fish, even at higher doses.

Regarding the wide variety of steroid compounds used for sex reversal in fish, different efficiencies arise from different affinities for their receptors, activities of the steroid-receptor complexes, and their metabolism (Devlin and Nagahama, 2002). Since all the steroids and the aromatase inhibitor used in the present study have proven their high potency for sex reversal in fish when administered during gonadal differentiation (Pandian and Sheela, '95; Devlin and Nagahama, 2002), particularly in tilapia (except 11KT that was used for the first time in Nile tilapia

Table 2. Effect of 17 α -ethynylestradiol (EE2) treatments applied in a single or double 4 hr-immersion at different moments of the embryonic development (1 and/or 24-hr post-fertilization, hpf) on the survival (at 8 days post-fertilization, dpf) and the sex ratio of XY Nile tilapia fry (* $P < 0.05$).

| Active substance | Concentration ($\mu\text{g l}^{-1}$) | Moment (hpf) | n XY spawns | % Survival (8 dpf) | | | | % Sex reversal | | | | | | |
|------------------|----------------------------------------|--------------|-------------|--------------------|-----|---------|----------|----------------|---------------|------|------|---------|----------|--------|
| | | | | Min | Max | Overall | χ^2 | P | Total n sexed | Min | Max | Overall | χ^2 | P |
| Control | 0 | 1 | 3 | 28 | 79 | 62 | 4.7 | 0.030 | 190 | 0.0 | 0.0 | 0.0 | | |
| EE2 | 1000 | 1 | 3 | 69 | 73 | 72* | 4.7 | 0.030 | 181 | 5.7 | 16.7 | 9.9* | 19.9 | <0.001 |
| EE2 | 2000 | 1 | 3 | 26 | 85 | 65 | 0.5 | 0.501 | 154 | 5.9 | 29.5 | 26.6* | 57.4 | <0.001 |
| Control | 0 | 24 | 7 | 35 | 86 | 75 | | | 500 | 0.0 | 0.0 | 0.0 | | |
| EE2 | 1000 | 24 | 3 | 38 | 71 | 60 | 3.5 | 0.063 | 127 | 43.6 | 66.7 | 48.0* | 65.7 | <0.001 |
| EE2 | 2000 | 24 | 8 | 32 | 86 | 76 | 0.2 | 0.629 | 553 | 2.0 | 91.0 | 48.6* | 326.7 | <0.001 |
| Control | 0 | 1 + 24 | 2 | 44 | 45 | 44 | | | 96 | 0.0 | 0.0 | 0.0 | | |
| EE2 | 1000 | 1 + 24 | 3 | 31 | 37 | 33* | 6.1 | 0.014 | 88 | 57.1 | 84.2 | 65.9* | 92.4 | <0.001 |
| EE2 | 2000 | 1 + 24 | 2 | 27 | 35 | 32* | 7.6 | 0.006 | 69 | 57.9 | 68.0 | 65.2* | 86.1 | <0.001 |

in our study) (Jalabert et al., '74; Potts and Phelps, '95; Kwon et al., 2000), the differences in efficiency of the treatments have to be searched in the developmental events targeted by the treatments, the endocrine and genetic mechanisms of sex determination and differentiation taking place during this precocious phase and the genotypic differences of tested fish.

As androgen (Golan and Levavi-Sivan, 2014) and estrogen (Kawahara and Yamashita, 2000; Singh, 2013) receptors are involved in hormone-induced sex reversal, their expression is a prerequisite to successfully override genetic sex determination by exogenous hormone exposure. In tilapia, the two androgen receptors (*ar1* and *ar2*) are expressed at a low level at the onset of gonadal development (from 9 dpf) and the three estrogen receptors (*esr1*, *esr2a*, and *esr2b*) at a high level, similarly in XX and XY (Ijiri et al., 2008). Before the formation of the gonad, the target of exogenous hormones in the mechanism of sex reversal could be the brain or the primordial germ cells and their environment (Kobayashi and Iwamatsu, 2005; Rougeot et al., 2008a). *Esr1* and *esr2a* are expressed in the brain of both XX and XY tilapia larvae from hatching (4 dpf). In contrast, the expression of androgen receptors is differentially initiated in XX and XY. *Ar1* transcripts are detected from 4 dpf in XY but not before 29 dpf in XX and *ar2* is expressed from 9 dpf in XY and from 4 dpf in XX (Sudhakumari et al., 2005). Even though both androgen and estrogen receptivities seem to be acquired early during fish development, a quantitative study of the expression of steroid receptors during tilapia embryogenesis and embryonic sex reversal would clarify their role in mediating the action of exogenous hormones, and we cannot exclude that a differential expression of the estrogen and androgen receptors may account for the differences in treatment potencies and genotype sensitivities. More specifically, the absence of one of the two *ar* in the brain of developing XX embryos could be responsible for their low susceptibility to androgen-induced sex reversal.

In natural sexual differentiation in tilapia, androgens do not appear to play an important role (Nagahama, 2005). The most potent natural androgen, 11KT, is not synthesized in the differentiating testis before 29 dpf (Ijiri et al., 2008). This suggests that androgen-signaling pathways may not be fully developed in embryos, explaining the low potency of androgens in embryonic sex-reversing treatments. In contrast, estrogens play a critical role in ovarian differentiation and the lack of estrogens during the period of gonad differentiation is responsible for testis development (Guiguen et al., '99; Kwon et al., 2000; Nagahama, 2005). Given that and the wide diversity of other estrogen functions during ontogenesis (Hao et al., 2013), estrogen-signaling pathways establish early during development and can therefore be used by exogenous hormones to override natural processes.

As the treatment of XX embryos with an aromatase inhibitor induced up to 10% sex inversion, it is proposed that aromatase and estrogens could play a role in sexual differentiation during embryogenesis. In Nile tilapia, brain aromatase was up-regulated

Table 3. Effect of 17 α -ethynylestradiol (EE2) treatments applied in a single 4 hr-immersion at different moments of the embryonic development (12, 18, 24, 36 hr post-fertilization, hpf) on the survival (at 8 days post-fertilization, dpf) and the sex ratio of YY Nile tilapia fry-(missing data; * $P < 0.05$).

| Active substance | Concentration ($\mu\text{g l}^{-1}$) | Moment (hpf) | n YY spawns | % Survival (8 dpf) | | | | % Sex reversal | | | | | | |
|------------------|----------------------------------------|--------------|-------------|--------------------|-----|--------|----------|----------------|---------------|-----|-----|---------|----------|--------|
| | | | | Min | Max | eOvall | χ^2 | P | Total n sexed | Min | Max | Overall | χ^2 | P |
| Control | 0 | 24 | 3 | - | - | - | - | - | 150 | 0.0 | 0.0 | 0.0 | 0.0 | >0.999 |
| EE2 | 2000 | 24 | 3 | - | - | - | - | - | 150 | 0.0 | 0.0 | 0.0 | 0.0 | >0.999 |
| Control | 0 | 24 | 4 | 38 | 77 | 64 | - | - | 353 | 0.0 | 0.0 | 0.0 | 0.0 | 0.096 |
| EE2 | 6500 | 12 | 4 | 10 | 42 | 24* | 320.2 | <0.001 | 128 | 0.0 | 1.8 | 0.8 | 2.8 | >0.999 |
| EE2 | 6500 | 18 | 4 | 25 | 80 | 60 | 3.5 | 0.062 | 337 | 0.0 | 0.0 | 0.0 | 0.0 | 0.311 |
| EE2 | 6500 | 24 | 4 | 38 | 78 | 61 | 2.5 | 0.115 | 344 | 0.0 | 1.0 | 0.3 | 1.0 | 0.30 |
| EE2 | 6500 | 36 | 4 | 38 | 80 | 62 | 0.8 | 0.369 | 333 | 0.0 | 1.2 | 0.3 | 1.1 | 0.30 |

in a dose-dependent manner in EE2-treated XY and YY embryos at 4 dpf (submitted manuscript), suggesting a possible modification of the brain sexual differentiation that could consequently play a role in the development of the sexual phenotype. Both aromatizable (MT) and non-aromatizable (dihydrotestosterone) androgens *in vitro* inhibited aromatase activity in ovarian microsomes of tilapia (Golan and Levavi-Sivan, 2014) suggesting that exogenous androgens administered during the differentiation of the gonad exerted an inhibitory action on aromatase activity which could be part of the mechanism of early masculinization. However, since masculinization rates were only 10% in XX spawns and no sex reversal was observed in YY spawns, changes in the expression or activity of brain aromatase could not be the sole factor involved in the mechanism of embryonic sex reversal.

Results of feminization in XY and the absence of susceptibility to EE2 embryonic treatments in YY can be explained by the presence on the X chromosome of a female inducer or alternatively the presence on the Y of a female repressor (or male inducer). In any case, the mechanism of embryonic sex reversal must rely on a balance between the action of the exogenous steroids and the upstream genetic factors involved in the sex-determining and differentiating cascade. Such a hormonal-genetic balance can be exemplified with *dmrt1* that is a conserved male marker specifically expressed in testes. Evidence of the importance of this gene in testicular differentiation was underlined by the discovery of its homolog *dmy* as the major sex-determinant in medaka (Matsuda et al., 2002). In tilapia, *dmrt1* is expressed specifically in the Sertoli cell lineage from 10 dpf (Ijiri et al., 2008; Kobayashi et al., 2008). It is up-regulated in the differentiating testis of MT-induced XX males and repressed in the differentiating ovary of EE2-induced XY females (Kobayashi et al., 2008), while XX fish carrying extra copies of *dmrt1* as a transgene develop into males (Nagahama, 2005). *Dmrt1* (or an homolog) is not the major sex determinant in tilapia (Volf et al., 2003) but a sex determinant upstream from *dmrt1* could be expressed during embryogenesis and prevents exogenous estrogen to override the genetic sex determinism when present in two copies.

In conclusion, as shown in a previous study using thermal treatments (Rougeot et al., 2008b), it is possible to artificially reverse the sex differentiation pathway of tilapia during embryogenesis, before the gonad differentiating period. Our results reinforced the hypothesis of an action of exogenous hormones on brain sexual differentiation or on primordial germ cells and their environment, and the important role of aromatase in sex differentiation mechanisms. Nevertheless, as estrogen treatments failed to reverse YY fish to females, the hypothesis of a sex determinant linked to the Y chromosome preventing the female pathway when present in two copies, or a gene present on the X chromosome needed for the development of a female phenotype, is suggested. The investigation of the genetic and

endocrine mechanisms involved in embryonic sex reversal would provide valuable insights on sex determination and early sex differentiation in the Nile tilapia.

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