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The Sensitive Period for Male-to-Female Sex Reversal Begins at the Embryonic Stage in the Nile Tilapia and is Associated With the Sexual Genotype

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SUMMARY

In this study, we sought to determine the mechanism of early sex reversal in a teleost by applying 4 hr feminization treatments to XY (17α-ethynylestradiol 2000 μg L^{-1}) and YY (6500 μg L^{-1}) Nile tilapia embryos on the first day post-fertilization (dpf). We then searched for changes in the expression profiles of some sex-differentiating genes in the brain (*cyp19a1b*, *foxl2*, and *amh*) and in sex steroids (testosterone, 17β-estradiol, and 11-ketotestosterone) concentrations during embryogenesis and gonad differentiation. No sex reversal was observed in YY individuals, whereas sex-reversal rates in XY progeny ranged from 0–60%. These results, together with the clearance profile of 17α-ethynylestradiol, confirmed the existence of an early sensitive period for sex determination that encompasses embryonic and larval development and is active prior to any sign of gonad differentiation. Estrogen treatment induced elevated expression of *cyp19a1b* and higher testosterone and 17β-estradiol concentrations at 4 dpf in both XY and YY individuals. *foxl2* and *amh* were repressed at 4 dpf and their expression levels were not different between treated and control groups at 14 dpf, suggesting that *foxl2* did not control *cyp19a1b* in the brains of tilapia embryos. Increased *cyp19a1b* expression in treated embryos could reflect early brain sexualization, although this difference alone cannot account for the observed sex reversal as the treatment was ineffective in YY individuals. The differential sensitivity of XY and YY genotypes to embryonic induced-feminization suggests that a sex determinant on the sex chromosomes, such as a Y repressor or an X activator, may influence sex reversal during the first steps of tilapia embryogenesis.

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INTRODUCTION

While the process of sex determination is relatively conserved and relies on a single pathway in the majority of mammals, fish utilize many different sex-determining systems that exist along a continuum ranging from pure genetic determination (chromosomal XY, ZW, or polygenic) to environmental determination (Devlin and Nagahama, 2002). In fish with genetic sex determination, a master sex-determining gene similar to the mammalian *SRY* (Sinclair et al., 1990) was characterized in a few species: *dmy* in the

Abbreviations: 11KT, 11-ketotestosetrone; dpf, days post-fertilization; E2, 17β-estradiol; EE2, 17α-ethynylestradiol; hpf, hours post-fertilization; P#, population number; T, Testosterone.; *amh*, anti-Müllerian hormone; *cyp19a1*, cytochrome P450 aromatase; *foxl2*, forkhead box l2 transcription factor

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medaka *Oryzias latipes* (Matsuda et al., 2002), *gsdf* in *Oryzias luzonensis* (Myosho et al., 2012), *amhy* in the Patagonian pejerrey *Odontesthes hatchery* (Hattori et al., 2012), *amhr2* in the tiger pufferfish *Takifugu rubripes* (Kamiya et al., 2012), and *sdY* in the rainbow trout *Oncorhynchus mykiss* (Yano et al., 2012). Of the most-important genes acting downstream of Sry analogs during sexual differentiation, *amh* (anti-Müllerian hormone) is associated with testis differentiation while *foxl2* (forkhead box l2 transcription factor) and *cyp19a1* (cytochrome P450 aromatase) are linked to ovarian development (Siegfried, 2010).

The role of *amh* during male differentiation in fish is still unclear as teleosts do not have Müllerian ducts. The finding of *amhy*, a duplicated gene on the Y chromosome of pejerrey, and its receptor *amhr2* as sex determinants reflects the importance of *amh* signaling in certain fish (Hattori et al., 2012; Kamiya et al., 2012). In tilapia, higher expression of *amh* is observed in the differentiating testis starting around 20 days post-fertilization (dpf) and remains at a higher level (Ijiri et al., 2008; Poonlaphdecha et al., 2011). Its dimorphic expression appears early in the brain of males, at the onset of gonad differentiation (10 dpf), suggesting an additional role in brain sexualization (Poonlaphdecha et al., 2011).

Foxl2, on the other hand, is expressed in a female-specific manner in the developing gonads of mammals, birds, reptiles (Loffler et al., 2003), and fish (Guiguen et al., 2010). In medaka, *foxl2* is up-regulated at the first stages of ovarian differentiation (Nakamoto et al., 2006). In tilapia, its sexually dimorphic expression was reported as early as 9 dpf, before any sign of histological differentiation (Ijiri et al., 2008). An in vitro study in tilapia identified *foxl2* as an important regulator of *cyp19a1* transcription (Wang et al., 2007); indeed these two genes are co-expressed in the differentiating ovaries of rainbow trout (Vizziano-Cantonnet et al., 2011) and tilapia (Wang et al., 2007).

Aromatase, encoded by *cyp19a1a* for the gonad form and *cyp19a1b* for the brain form (Tchoudakova and Callard 1998; Kwon et al., 2001), is responsible for estrogen synthesis, and therefore plays a key role in sexual steroidogenesis and gonad differentiation in fish (see reviews by Diotel et al., 2010 and Guiguen et al., 2010). For example, administration of estrogens to genetic males during the sex differentiation period induced feminization of the gonads in different species (Piferrer, 2001; Devlin and Nagahama, 2002) whereas treatment of fry with aromatase inhibitor leads to masculinization in genetic females (Piferrer et al., 1994; Afonso et al., 2001; Uchida et al., 2004). In rainbow trout, c*yp19a1a* is up-regulated in the differentiating ovary (von Schalburg et al., 2010) while in tilapia, expression of *cyp19a1a* is up-regulated in female gonads from 9 dpf onwards (D'Cotta et al., 2001; Ijiri et al., 2008; Poonlaphdecha et al., 2013) and *cyp19a1b* is expressed in the brain very early during ontogenesis, with no difference in expression profiles between sexes (Kwon et al., 2001). Although the regulatory pathways of *cypa19a1a* and *cyp19a1b* have their specificities, several authors suggested a possible role for brain aromatase during brain and the gonad sexualization in gonochoristic teleosts (Tsai et al., 2003; Blázquez and Somoza, 2010; Le Page et al., 2010).

Nile tilapia has an XX/XY sex determination system that can be overridden by exogenous steroids and high temperature (masculinizing effect above 32°C) (Jalabert et al., 1974; Baroiller et al., 1995, 1999). The sensitive period extends from 10 dpf (yolk-sac resorption) to 25–30 dpf (formation of an ovarian cavity or an intratesticular efferent duct) (Kobayashi et al., 2008). Temperature-induced masculinization up-regulates gonad expression of *amh* while repressing both *foxl2* and *cyp19a1a* expression as well as brain aromatase activity (D'Cotta et al., 2001; Poonlaphdecha et al., 2013). In contrast, estrogen-feminizing treatments up-regulate the expression of both *cyp19a1* genes (Kobayashi et al., 2003). Several authors suggest that tilapia also possesses a second sensitive window for sex determination, starting earlier in the embryonic phase. Rosenstein and Hulata (1992) attempted to feminize Mozambique tilapia (*O. mossambicus*) using short estrogen (17β-estradiol, progesterone, and flutamide) immersions of freshly fertilized eggs, but found a similar number of females in treated and control groups. Rougeot et al. (2008a,b) showed that thermal (>34°C) and hormonal (17α-methyltestosterone, 17α-ethynylestradiol) treatments of embryos from 12 hr post-fertilization (hpf) to hatching induced, respectively, up to 27% and 68% sex reversal in *O. niloticus*. They suggested that early sex reversal treatments could act on the development of primordial germ cells, apparent as early as 46 hpf, and/or somatic cells of the future gonad, or could influence brain sexualization since the brain starts to differentiate at 31 hpf (Morrison et al., 2001). A separate model that must be considered when interpreting how these early hormonal treatments affect the sex-reversal mechanism is the possible accumulation of hormones in the embryo and the vitellus, which could lead to a delayed effect on embryogenesis such that the hormones act directly on the developing gonad (Piferrer and Donaldson, 1994). Characterization of the uptake and clearance kinetics of 17α-ethynylestradiol (EE2) administered to XY embryos following Rougeot's procedure showed that the whole-body concentration of EE2 at the onset of gonad differentiation (10 dpf) was still very high (unpublished data). Such delayed and prolonged hormonal supply makes the identification of the period of hormone action and target structure more difficult because of its ambiguity.

The present study aimed to substantiate the existence of a precocious sensitive period for sex reversal in Nile tilapia, before the development of a gonadal primordium tissue, and to explore the mechanism(s) of sex reversal during this period. We used short EE2 immersion treatments applied at early embryonic stages to switch the phenotypic sex of XY Nile tilapia. EE2 uptake and clearance were followed to confirm early action of the exogenous hormone. Similar treatments were applied to YY embryos to test the influence of sexual genotype on sex-reversal sensitivity and on the sex determination process. Sex-reversal mechanisms and the hypothetical upstream role of the brain in the sex determination process were investigated through the measurements of the natural sexual steroids (testosterone, 17β-estradiol, and 11-ketotestosterone) and the expression of three main sex-differentiating genes (*cyp19a1b*, *amh*, and *foxl2*) in the heads of treated embryos and juveniles.

RESULTS

Growth, Survival, and Sex-Ratios

Immersion treatments did not significantly affect the growth of XY progeny during the whole experimental period (Fig. 1A). At 35 dpf, mean body weight of the three groups were: 651 ± 14 mg for the group immersed at 1 dpf for 4 hr in a dose of 2000 μg EE2 L^{-1} (XY2000), 643 ± 12 mg for the controls (XYC) immersed in ethanol 1:1000, and 649 ± 15 mg in the group immersed at 10 dpf for 4 hr in a dose of 20 μg EE2 L^{-1} (XY20).

Growth was very similar between the YY progeny as well (Fig. 1B), and immersion treatment did not affect the mean body weight of the fish immersed at 1 dpf for 4 hr in a 6500 μg EE2 L^{-1} solution (YY6500) or in the controls (YYC) immersed at 1 dpf for 4 hr in ethanol 1:1000. In the group fed from 10–40 dpf with 500 mg EE2 kg^{-1} food (YYD), which served as a positive control for sex reversal, growth slowed down after 19 days of treatment (28 dpf), resulting in a significantly different mean body weight from both YYC and YY6500 at 35 and 42 dpf. At the end of the experiment (42 dpf), the mean body weights were respectively 838 ± 73, 864 ± 80, and 433 ± 64 mg in YYC, YY6500, and YYD progeny.

Survival rates in the XY progenies ranged from 11–29% in XYC, from 16–34% in XY2000, and from 24–50% in XY20 groups (Table 1). Mean values were not significantly different between XYC and XY2000, but the mean survival rate was significantly higher in the XY20 group than in the controls. In YY progeny, survival rates ranged from 38–58% in YYC, from 25–45% in YY6500, and from 53–71% in YYD groups. The mean survival rate was significantly higher in YYD compared to YYC and YY6500 progeny.

Sex-ratio analysis revealed the expected absence of females in the control groups (XYC and YYC) (Table 1). In XY individuals, the efficiency of EE2 immersion treatments differed between progeny. The proportion of females in XY2000 ranged from 0.9–60.6%. Except for population 4 (P4) progeny, immersion treatment with a dose of 2000 μg L^{-1} EE2 for 4 hr applied to 1 day-old embryos (XY2000) significantly skewed the sex ratios toward females, with the highest values for P2 (60.6%) and P5 (23.0%). Immersion treatment of 10-days-old larvae at 20 μg L^{-1} EE2 resulted in significant sex reversal only in these two progeny populations, at 8.6% and 4.0% of females for P2 and P5, respectively, suggesting their increased sensitivity to steroid feminization. In YY progeny, however, EE2 immersion treatment at 1 dpf did not lead to any significant sex reversal, although these populations were clearly susceptible as dietary EE2 treatment (from 10–40 dpf) achieved sex reversal efficiencies of 83.7–100%.

EE2 Uptake and Clearance

The EE2 used for the feminization treatment of XY embryos (XY2000 group, 2000 μg L^{-1} at 1 dpf) passed through the chorion and rapidly accumulated in the eggs, resulting in a mean concentration of 18929 ± 3565 ng g^{-1} shortly after the immersion (Fig. 2A). This surge represented a 10-fold bioconcentration of the hormone compared to the initial exposure dosage. Clearance kinetics were rapid, as the concentration dropped to 4798 ± 465 ng g^{-1} by 4 dpf and returned to a value (1.41 ± 1.89 ng g^{-1}) statistically similar to the control at 21 dpf (Fig. 2A and B).

The 20 μg L^{-1} EE2 dose used for the 4 hr treatment at 10 dpf (XY20) was chosen in order to reach the same tissue concentration as the XY2000 group, allowing us to test if the XY2000 treatment was active before 10 dpf. EE2 tissue concentration of XY20 was 3.5-fold higher than in XY2000 (3975 ± 995 and 1146 ± 153 ng g^{-1} respectively) at 10 dpf following treatment (Fig. 2B). One day after the treatment, however, the EE2 level of XY20 dropped to 397 ± 39 ng g^{-1} and continued to decrease faster than in XY2000 individuals. At 14 dpf, the values of EE2 concentration in XY2000 (28 ± 12 ng g^{-1}) and XY20 (10 ± 5 ng g^{-1}) were not significantly different, although they remained significantly higher

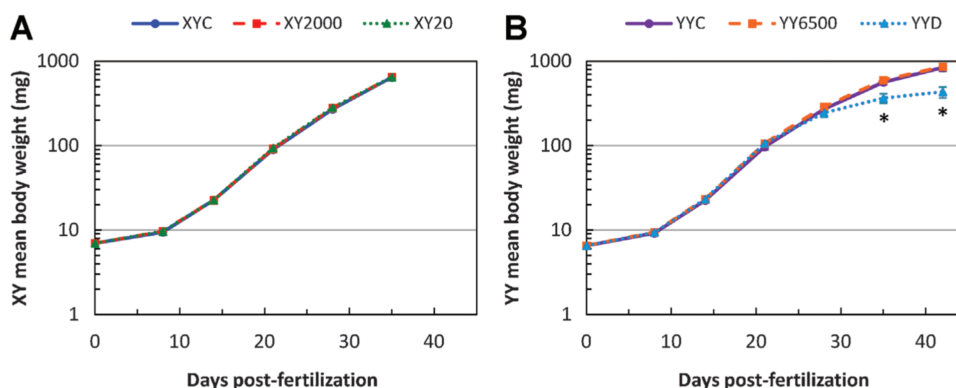

Figure 1. Mean growth curves of tilapia progeny during the experimental period in (A) XY (n = 5) until 35 dpf and (**B**) YY (n = 4) until 42 dpf. XYC, XY control group; XY2000, XY batch that underwent an EE2 immersion treatment (2000 μg L^{-1}) for 4 hr at 1 dpf; XY20, XY batch immersed in EE2 at 20 μg L^{-1} for 4 hr at 10 dpf; YYC, YY control group. YY6500, YY batch immersed in EE2 at 6500 μg L^{-1} for 4 hr at 1 dpf; YYD, YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10–40 dpf. *$P < 0.05$.

TABLE 1. Survival Rates in 5 XY (35 dpf; P1 to P5) and 4 YY (42 dpf; P6 to P9) Progeny, and Sex-Ratios (% Females) at 90 dpf for Tilapias Submitted to EE2 Feminization Treatments

		XYC	XY2000	XY20		YYC	YY6500	YYD
Survival (%)		24.5 ± 3.5^{a}	27.7 ± 3.2^{ab}	40.6 ± 4.6^{b}		48.7 ± 4.4^{a}	37.9 ± 4.7^{a}	66.5 ± 4.5^{b}
% females (n sexed)	P1	0.0^{a} (53)	11.8^{b} (68)	0.0^{a} (66)	P6	0.0^{a} (103)	0.9^{a} (106)	99.1^{b} (113)
	P2	0.0^{a} (125)	60.6^{c} (109)	8.6^{b} (93)	P7	0.0^{a} (100)	0.8^{a} (124)	100.0^{b} (100)
	P3	0.0^{a} (110)	6.3^{b} (111)	0.0^{a} (101)	P8	0.0^{a} (100)	0.0^{a} (118)	99.0^{b} (100)
	P4	0.0^{a} (106)	0.9^{a} (106)	0.0^{a} (104)	P9	0.0^{a} (103)	0.0^{a} (58)	83.7^{b} (92)
	P5	0.0^{a} (100)	23.0^{c} (100)	4.0^{b} (101)				
	Mean	0.0 ± 0.0^{a}	20.5 ± 10.7^{c}	2.5 ± 1.7^{b}	Mean	0.0 ± 0.0^{a}	0.4 ± 0.3^{a}	95.5 ± 3.9^{b}

XYC, XY control group; XY2000, XY submitted to an EE2 immersion treatment (2000 μg L^{-1}) for 4 hr at 1 dpf; XY20, XY immersed in EE2 at 20 μg L^{-1} for 4 hr at 10 dpf; YYC, YY control group; YY6500, YY immersed in EE2 at 6500 μg L^{-1} for 4 hr at 1 dpf; YYD, YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10–40 dpf. Different superscript letters (a, b, or c) indicate significant differences ($P < 0.05$).

than the controls. As in XY2000, the hormone level in XY20 returned to a basal level close to 0 ng g^{-1} and was statistically similar to the controls at 21 dpf (0.55 ± 0.67 ng g^{-1}).

In YY feminization experiments at a high concentration of 6500 μg L^{-1} (YY6500), EE2 uptake and clearance followed the same profile as in XY2000, with a 10-fold bioconcentration of the hormone in the tissue immediately after immersion (Fig. 2C) and a maximum mean concentration of 62755 ± 3691 ng g^{-1}. EE2 level decreased thereafter to 6703 ± 1789 ng g^{-1} at 10 dpf. At 14 dpf, the concentration in YY6500 (28 ± 7 ng g^{-1}) was equal to that measured in XY2000, and returned to a level (0.26 ± 0.03 ng g^{-1}) statistically similar to the control at 21 dpf (Fig. 2D). In the dietary EE2 groups (YYD) given a dose of 500 mg EE2 kg^{-1} in their food from 10–40 dpf, the EE2 tissue concentration slowly increased to 166 ± 44 ng g^{-1} at 14 dpf, 442 ± 40 ng g^{-1} at 21 dpf, and reached a maximum of 1792 ± 295 ng g^{-1} at the end of the treatment period (40 dpf) (Fig. 2D). As soon as the hormonal treatment ended, the concentration decreased rapidly to 139 ± 21 ng g^{-1} at 42 dpf.

Testosterone, 17 β-Estradiol, and 11-Ketotestosterone Profiles

Eggs at 0 dpf contained high levels of testosterone (T) and 17 β-estradiol (E2) in both XY (Fig. 3A and C) and YY (Fig. 3B and D) progeny. The maximal values were measured at 1 dpf (except for T in YY): 58 ± 5 ng T g^{-1} and 45 ± 11 ng E2 g^{-1} in XYC; 37 ± 9 ng T g^{-1} and 67 ± 6 ng E2 g^{-1} in YYC. After 1 dpf, concentrations of T and E2 decreased until 10–14 dpf,

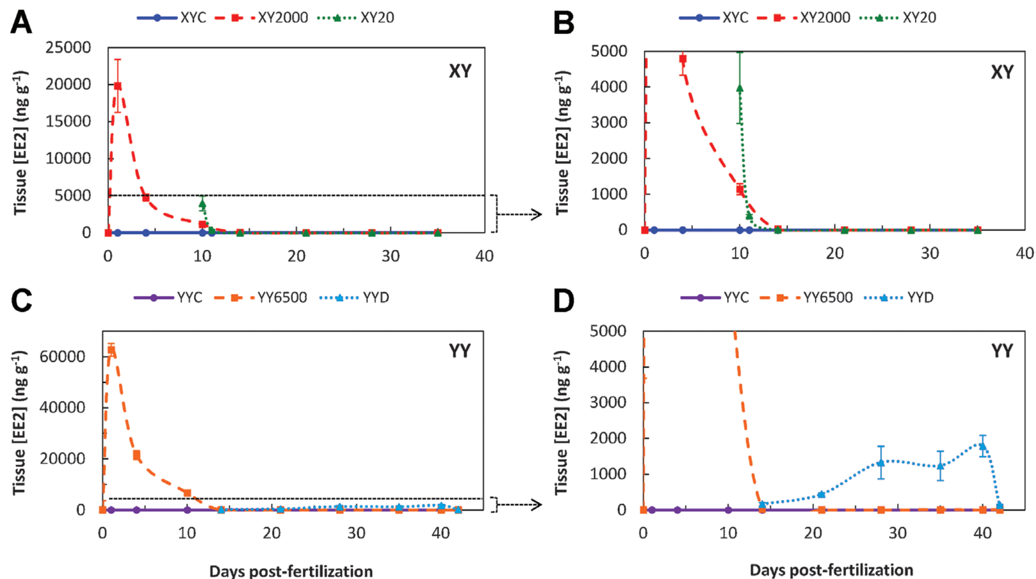

Figure 2. Mean 17α-ethynylestradiol (EE2) tissue concentration in (**A**, **B**) XY (n = 5 progenies) and (**C**, **D**) YY (n = 4) progeny of Nile tilapia submitted to feminization treatments. **A** and **C**: complete profiles; **B** and **D**: data between 0 and 5000 ng g^{-1}. XYC, XY control group; XY2000, XY submitted to an EE2 immersion treatment (2000 μg L^{-1}) for 4 hr at 1 dpf; XY20, XY immersed in EE2 at 20 μg L^{-1} for 4 hr at 10 dpf. YYC, YY control group; YY6500, YY immersed in EE2 at 6500 μg L^{-1} for 4 hr at 1 dpf; YYD, YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10–40 dpf.

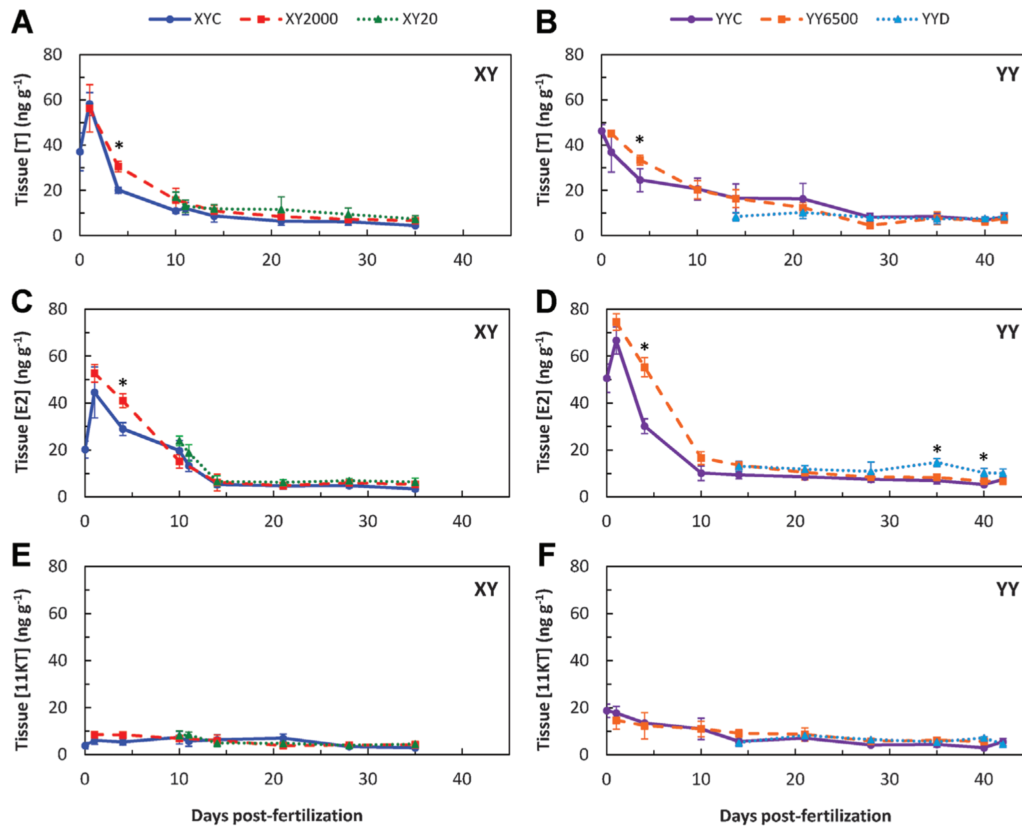

Figure 3. **A**, **B**: Mean testosterone (T), **C**, **D**: 17β-estradiol (E2), and **E, F**: 11-ketotestosterone tissue concentration in XY ($n = 5$) and YY ($n = 4$) progenies of Nile tilapia submitted to feminization treatments. XYC, XY control group; XY2000, XY submitted to an EE2 immersion treatment (2000 μg L^{-1}) for 4 hr at 1 dpf; XY20, XY immersed in EE2 at 20 μg L^{-1} for 4 hr at 10 dpf. YYC, YY control group; YY6500, YY immersed in EE2 at 6500 μg L^{-1} for 4 hr at 1 dpf; YYD, YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10–40 dpf. *$P < 0.05$.

and remained at a low level (between 5 and 15 ng g^{-1}) until the end of the experimental period.

For both XY and YY groups, individuals that received EE2 immersion treatment at 1 dpf (XY2000 and YY6500) showed a significantly increased level of T and E2 at 4 dpf compared to controls (Fig. 3A–D). No other significant differences were observed between controls and immersion-treated groups. E2 concentrations significantly increased in dietary EE2-treated YY progeny at 35 and 40 dpf (Fig. 3D).

The profile of 11-ketotestosterone (11KT) was different than T or E2. In XY individuals (Fig. 3E), mean levels of 11KT were low and constant from 0–35 dpf and did not exceed 7 ng g^{-1}. YY eggs, however, contained a higher concentration of 11KT at 0 dpf (19 ± 3 ng g^{-1}) (Fig. 3F), but decreased thereafter until 10 dpf, settling at a similar level to the XY group. No significant difference was observed in 11KT levels between control and treated groups among any of the genotypes.

Overall, no correlation was observed between steroid levels in eggs at 0 dpf and sex reversal rates.

Expression Analysis of Sex-Differentiating Genes

Early feminization treatments caused a strong and significant increase in *cyp19a1b* expression in the heads of tilapia embryos three days after the treatment (at 4 dpf) (Fig. 4A and B). In the XY2000 EE2-treated group, we observed a 2.4-fold change in the relative expression of this gene ($39.1 \pm 2.7\%$) compared to XYC controls ($16.1 \pm 1.2\%$) (Fig. 4A). Brain aromatase expression was even higher in immersion-treated YY progeny (Fig. 4B), with a 3.5-fold increase in relative expression seen in the YY6500 ($64.7 \pm 5.6\%$) compared to YYC controls ($18.4 \pm 1.3\%$). No significant difference was observed between XY and YY controls. On the other hand, 4 dpf expression levels of *amh* and *foxl2* were very low (Fig. 4A and B), and were not affected by the hormonal immersion treatments or by the genotype ($P > 0.05$).

None of the three analyzed genes showed a significant difference in expression levels between control and EE2-treated groups or between genotypes in the heads of 14 dpf fry (Fig. 4C and D). Compared to 4 dpf, the relative expression of *cyp19a1b* was lower in all the groups (XYC, $10.2 \pm 1.6\%$; XY2000, $11.9 \pm 3.7\%$; YYC, $7 \pm 1.3\%$; YY6500, $13.2 \pm 2.4\%$). Both *amh* and *foxl2* expressions, however, were up-regulated at this age. From 4–14 dpf, *amh* expression increased from 1.0 ± 0.5–$16.2 \pm 2.3\%$; from 0.5 ± 0.2–$12.9 \pm 3.1\%$; from 1.1 ± 0.3–$30.4 \pm 15.6\%$; and from 1.7 ± 0.4–

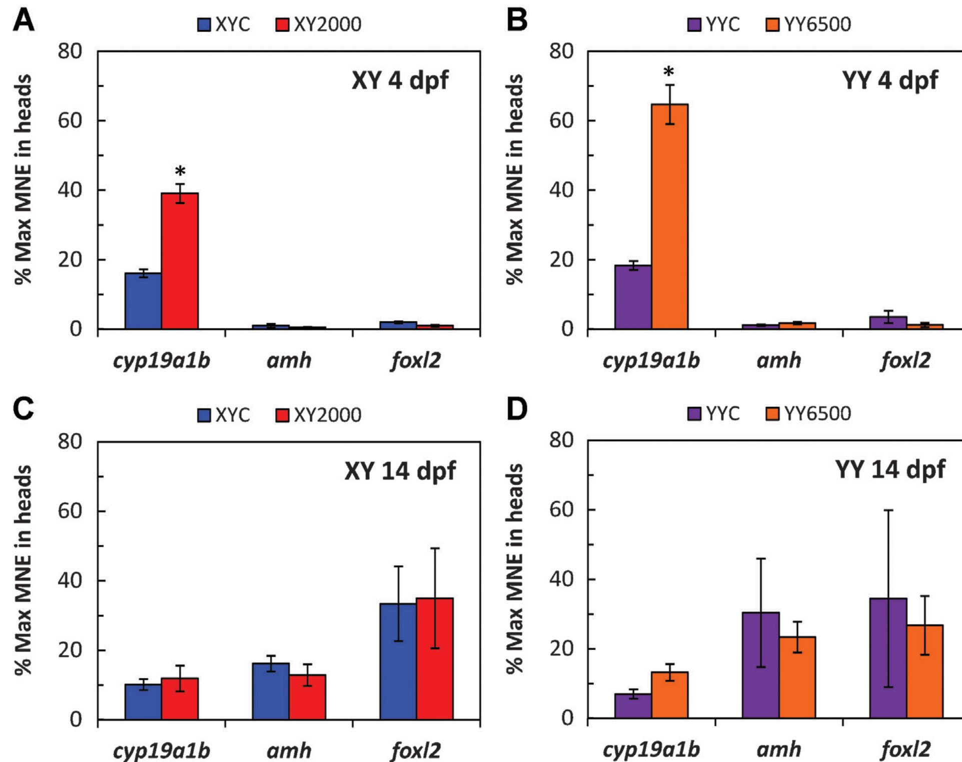

Figure 4. Mean relative expression of *cyp19a1b*, *amh*, and *foxl2* at 4 dpf (**A**, **B**) and 14 dpf (**C**, **D**) in heads of XY (n = 5) and YY (n = 4) progeny of Nile tilapia submitted to early-feminization treatment. XYC, XY control group; XY2000, XY submitted to an EE2 immersion treatment (2000 μg L^{-1}) for 4 hr at 1 dpf; XY20, XY immersed in EE2 at 20 μg L^{-1} for 4 hr at 10 dpf. YYC, YY control group; YY6500, YY immersed in EE2 at 6500 μg L^{-1} for 4 hr at 1 dpf; YYD, YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10–40 dpf. *$P < 0.05$.

23.4 ± 4.4% in XYC; XY2000 YYC; and YY6500 groups, respectively. *Foxl2* expression increased from 2.0 ± 0.3–33.4 ± 10.7%; from 1.0 ± 0.3–35.0 ± 14.3%; from 3.5 ± 1.7–34.5 ± 25.5%; and from 1.2 ± 0.6–26.8 ± 8.5% in XYC; XY2000 YYC; and YY6500 groups, respectively.

No correlation was observed between changes in gene expression levels in control versus treated groups and sex reversal rates.

DISCUSSION

A 4 hr-immersion treatment in 2000 μg EE2 L^{-1} at 1 dpf induced up to 60% feminization in XY fish, confirming the existence of an early sensitive period for sex reversal during sex determination in the Nile tilapia. To our knowledge, this is the first time that early hormonal treatment was shown to induce sex reversal in tilapia. Similar feminizing treatments were performed on *O. mossambicus* embryos by Rosenstein and Hulata (1992), but failed to induce a sex-reversed phenotype. Our first work (Rougeot et al., 2008a) reported that immersion treatment in 100–500 μg EE2 L^{-1} from 12 hpf to 5 dpf induced 55–68% sex reversal in XY individuals, but the duration of the treatment and the consequent persistence of exogenous estrogen in the developing fry (more than 5000 ng g^{-1} at 10 dpf; unpublished data) left some uncertainty about the actual period of exogenous estrogen activity. Here, we shortened the treatment period and used a higher EE2 concentration in order to focus on treating during the embryonic stage, thereby avoiding a delayed effect due to the accumulation of hormone after 10 dpf. As demonstrated by the low deviation in sex ratio induced by treating individuals at 10 dpf, residual hormone from the early treatment at 1 dpf could not be responsible for the observed sex-reversed effect; the sex reversal phenotype is more likely caused by a mechanism operating before 10 dpf.

Complete sex reversal of *O. niloticus* XY fry, via an immersion treatment at the onset of gonad differentiation, was also achieved by Kobayashi et al. (2003) using similar or higher EE2 concentrations than we did (from 10–1000 μg L^{-1}) for a period of 3 days (from 8–10 dpf). An inverse relationship between dose and treatment duration has been reported for sex reversal (Piferrer, 2001), so it is likely that obtaining 60% sex reversal requires a higher estrogen intake at 10 dpf than the residual concentration measured in individuals treated at 1 dpf. The increase in T and E2 concentrations and the up-regulation of *cyp19a1b* expression induced by the early treatment were observed at 4 dpf but was not sustained by 14 dpf, suggesting that hormonal treatment acted only briefly on these parameters. The

hormone-sensitive period of sex determination in embryos corresponds to the early thermo-sensitive period for masculinization that was determined by Rougeot et al. (2008b). As temperature likely does not act through accumulation or provide a substantial delayed effect, these two studies are consistent with the existence of an embryonic sensitive period during which sex can be reversed in Nile tilapia.

A brief hormone exposure avoided the drawback of reduced survival rates induced in thermal treatments (Rougeot et al., 2008b; Wessels et al., 2011) or by longer exposures (Rosenstein and Hulata, 1992; Rougeot et al., 2008a). Survival-rate values, however, have to be cautiously interpreted from this study; these rates were likely underestimated due to the timed sampling process, which introduced an important bias that more severely affected groups in which the number sampled represents a high proportion of the total number of fish (XY2000, XYC, YY6500, and YYC groups). Given the similar survival rates estimated in XYC and XY2000, on the one hand, and in YYC and YY6500, on the other hand, plus the low mortality observed during the experimental period, we can conclude that brief (4 hr) exposure of tilapia embryos to EE2 (up to 6500 $\mu g\,L^{-1}$) does not affect their survival. Short immersions also did not adversely affect growth during the experimental period. This result contrasts with the impaired growth reported by Shved et al. (2008) following long-term exposure (from 10–100 dpf) of XY tilapia to environmentally relevant EE2 concentrations (5–25 $ng\,L^{-1}$). Growth was also reduced in dietary EE2-treated XY tilapia (at 125 $mg\,kg^{-1}$ food) from 90 dpf, that is, 50 days after the end of the treatment (Shved et al., 2007); we similarly observed a 50% growth reduction in YY fry fed with an EE2-supplemented diet (500 $mg\,kg^{-1}$ food) at the end of the treatment (42 dpf). This early decrease in growth might be attributed to a toxic effect of EE2 that arises with higher doses.

Early sex reversal in embryos by brief hormonal exposure was also observed in medaka (Kobayashi and Iwamatsu, 2005; Iwamatsu et al., 2005; 2006a). As medaka and Nile tilapia undergo similar morphogenesis during sex differentiation (Siegfried, 2010) and both present an early, sensitive sex-determining period long before gonadal differentiation, these two species are interesting models of gonochoristic teleosts for studying upstream sex-determining mechanisms. Unlike in medaka, however, no major sex determinant that is similar to the medaka *dmy* gene nor the involvement of primordial-germ-cell proliferation or their surrounding cells in the mechanism of early sex reversal (Kobayashi et al., 2004) has yet been defined in the Nile tilapia (Cnaani et al., 2008; Palaiokostas et al., 2013). On the other hand, Rougeot et al. (2008a) proposed and D'Cotta et al. (2001) provided evidence for a role of the brain in the sex-differentiation cascade. To explore this paradigm, we searched for sex-differentiating genes differentially expressed in the head of estrogen-immersed embryos.

Early estrogen treatments affected brain differentiation, as *cyp19a1b* was clearly up-regulated at 4 dpf in those embryo heads exposed to EE2. *Cyp19a1b* is very sensitive to exogenous estrogens due to the presence of a conserved estrogen responsive element in its promoter (Diotel et al., 2010). Estrogens and high aromatase activity are recognized to play an important role in neurogenesis, although their role in brain sexual differentiation is still unclear (Le Page et al., 2010). *Cyp19a1b* is expressed in the brain early in development in different species, with a clear sexually dimorphic expression before the onset of gonad morphological differentiation reported in the rainbow trout (Vizziano-Cantonnet et al., 2011). In the pejerrey, *cyp19a1b* was not differentially expressed before the onset of sex differentiation at either female- or male-promoting temperatures; in adults, however, E2 treatments caused *cyp19a1b* up-regulation together with the estrogen receptors *er*α and *er*β (Strobl-Mazzulla et al., 2008). No sex differences for *cyp19a1b* were found during development in medaka, although higher levels were observed in adult females (Okubo et al., 2011). In the Nile tilapia, the expression of the two genes coding for aromatase is initiated in embryos at 3–4 dpf (Kwon et al., 2001). During gonad differentiation, high expression level of the ovarian form of *cyp19a1b* (from 9 dph) is found in female gonads (D'Cotta et al., 2001; Ijiri et al., 2008) while the brain form is expressed at the same level in XX and XY brains (Kwon et al., 2001), suggesting that in tilapia it plays a role in neurogenesis rather than in sexual differentiation.

Temperature-dependent sex reversal is accompanied by a decrease in brain aromatase activity during the sex-differentiating period (D'Cotta et al., 2001), with higher brain aromatase activity in XX females than in XY males under normal thermal conditions. This observation, together with our results, suggest that a role for the brain and particularly for brain aromatase in gonad sex differentiation cannot be ruled out, and that post-transcriptional control could be responsible for regulating sex-specific aromatase activity (Balthazart et al., 2011). In tilapia, sex-specific expression of *cyp19a1b* may be restricted to certain nuclei and not be readily observable. Comparison between XY and YY genotypes showed that this increase in gene expression was dependent on EE2 dose, and was also associated with an increase in both T and E2 concentrations. While the increase in E2 synthesis was probably a result of the elevated *cyp19a1b* levels induced by exogenous EE2, the increase in T suggests that estrogen treatment also up-regulated the expression or activity of other steroidogenic enzymes long before the gonad is formed. Despite these associations, the up-regulation of *cyp19a1b* in treated embryos cannot be entirely responsible for the sex-reversal effect seen in the present study as no sex reversal was observed in YY progeny. If the brain is involved in the mechanism of early sex reversal, and consequently in early sex determination, this mechanism should be more complex and mostly likely require additional factors beyond aromatase.

To investigate one of the possible regulators of *cyp19a1b* expression, we measured the early expression of *foxl2* in the brain of normal and sex-reversed XY and YY tilapias. Transactivation studies have shown that the transcription factor *foxl2*, together with steroidogenic factor 1 (*sf1*), can activate the transcription of *cyp19a1a* by binding to its promoter (Wang et al., 2007). In tilapia gonads, *foxl2* is co-expressed

at the initiation of the ovarian differentiation, suggesting that it regulates in vivo *cyp19a1a* expression (Wang et al., 2007; Ijiri et al., 2008)—although temporal co-expression was not evident in other tilapia strains and temperature-induced masculinization did not suppress expression of both genes simultaneously (Poonlaphdecha et al., 2013). Our present results also suggest that brain-expressed *foxl2* does not regulate *cyp19a1b* expression, and does not participate in early sex reversal. We found very low or no expression of *foxl2* in the embryonic head of XY controls and in EE2-treated embryos, whereas *cyp19a1b* was markedly and differentially expressed between the two groups. As *foxl2* expression is strongly induced by estrogens in the gonad (Baron et al., 2004; Wang et al., 2007), we postulate that this gene was totally repressed or not yet transcribed in the brain of tilapia embryos at the stage analyzed as *foxl2* was expressed during gonad differentiation on 14 dpf at a similar level in both XY and YY fish and those treated with EE2. Consistent with our findings, *foxl2* is not the only trigger regulating *cyp19a1b* in the brain in rainbow trout (Vizziano-Cantonnet et al., 2011), and a cooperative regulation of *cyp19a1b* by *foxl2* and the nuclear receptor *ftz-f1* has been demonstrated in *Clarias gariepinus* (Sridevi et al., 2012). More investigations are needed to understand the role of *foxl2* in the brain of teleosts.

Amh was another candidate cerebral genetic factor that could be involved in early sexual reversal. In different species, *amh* and *cyp19a1a* have reciprocal expression profiles in the differentiating gonad (Wang and Orban 2007; Fernandino et al., 2008). In Nile tilapia, Poonlaphdecha et al. (2013) suggested that *cyp19a1a* inhibits *amh* expression in the gonads of genetic females. *Amh* is down-regulated by estrogens (Schulz et al., 2007; Fernandino et al., 2008) in the gonad, and sexually dimorphic expression levels are observed between genetic males and females in the tilapia brain at 14 dpf (Poonlaphdecha et al., 2011). In our study, *amh* expression was totally repressed in embryos independent of EE2 exposure. As observed for *foxl2*, *amh* expression was initiated later in the brain and showed no difference between controls and EE2 sex-reversed fish. This suggests that the expression level of *amh* could be related to the sex genotype, and that a factor linked to the Y chromosome may control its expression regardless of the sex phenotype.

The sensitivity of XY progeny to EE2 treatments varied widely among individuals, as demonstrated by the sex-reversal rates ranging from 0–60%. This variability could be related to the differential expression of sex-determining genes or to the endogenous steroid balance during ontogenesis and maternal steroid inheritance. In tilapia, estrogens are required for ovarian differentiation (Kobayashi et al., 2003; Nakamura, 2010) and are also involved in neurogenesis and brain sexualization, as suggested by the early expression of brain aromatase (Diotel et al., 2010; Le Page et al., 2010). Steroid metabolism is initiated early in embryogenesis as maternal hormones are metabolized during the first days of development (Hines et al., 1999). We therefore hypothesize that the steroid content of freshly fertilized eggs is maternally inherited and may influence the sex-determining mechanism. Yet, we did not find a correlation between steroid content (T, E2, and 11KT) in freshly ovulated eggs and sex-reversal rates. Deviation from a 1:1 sex ratio in normal crosses (Lester et al., 1989) and heritable differences in temperature-related sex influences (Baroiller et al., 2009; Wessels and Hörstgen-Schwark, 2011) demonstrate that sex determination is multifactorial in tilapia; we suggest that such factors could further influence the responsiveness to early hormonal treatment.

If egg steroid content is not a determinant of sexual development, we postulate that a genetic determinant present on the sex chromosomes is responsible for the absence of early sex reversal in YY. For example, there could be a feminizing factor linked to the X chromosome that is necessary to elicit a response to the estrogen treatment, or alternatively a masculinizing factor linked to the Y chromosome wherein the presence of two copies represses or disables any feminizing effect of the exogenous hormone. The absence of sex reversal in YY is nevertheless a striking and very interesting result as it suggests that an upstream genetic determinant linked to the sex chromosomes can influence sex determination in the first days of the embryogenesis, long before the earliest known evidence of a gonadal differential gene expression (9 dph) (Ijiri et al., 2008).

In summary, we demonstrated for the first time that brief EE2 treatments during embryogenesis are effective in reversing XY tilapias towards a female phenotype, highlighting the existence of an early sensitive period for sex reversal during tilapia sex determination encompassing embryonic and larval development, prior to any sign of gonad differentiation. This early-embryo induced feminization was ineffective in YY fish, suggesting that a Y-linked repressor may be modulator or alternatively that an activator on the X chromosome might be needed. Our embryonic feminizing procedure thus constitutes a new tool for identifying major, upstream sex determinants in the Nile tilapia.

MATERIAL AND METHODS

Fish Stock Housing, Reproduction, and Juvenile Rearing

Nile tilapia *O. niloticus* of the Lake Manzala strain were from the Research and Education Center in Aquaculture (CEFRA), University of Liège, Belgium. All-male XY progeny were obtained by artificial reproduction of YY males with XX females, and supermale YY progenies from YY males mated with YY females, as described in Gennotte et al. (2012).

After artificial fertilization, eggs were weighed and counted. Each progeny was divided into three batches (for the XY group: controls, a 4-hr-treated group at 1 dpf, and a 10 dpf-treated group; for the YY group: controls, a 4-hr-treated group at 1 dpf, and a 30-day diet-treated group) and incubated in 1.5 L Zug bottles at 27 °C. Fertilization rates were evaluated on 100 eggs after the first mitotic cleavage (2 hpf) (Morrison et al., 2001). Hatching rates were assessed by counting all fish from each batch at 4 dpf.

Larvae were transferred at 8 dpf into 50-L aquaria (mean stocking density ± standard deviation of 5.4 ± 1.7 fish L^{-1}) and feeding started at 10 dpf. In order to determine the food ratio and follow the growth and survival of each batch, the number of individuals and total biomass were recorded every week from 8 dpf to 35 dpf (XY) or 42 (YY) dpf. Homogenization of fish growth between treated and control groups, and among progeny, was essential to standardize the developmental speed and the post-treatment kinetics of EE2 clearance. Fish were fed close to satiation, and food ratios were adjusted to assure the same growth in all batches. Food distribution was performed six times a day with a commercial tilapia diet (47% proteins, 8% lipids) (Coppens, The Netherlands). Experiments were carried out according to the guidelines of the University of Liège ethical committee and the European animal welfare recommendations.

17α-Ethynylestradiol Solutions

17α-ethynylestradiol (Sigma–Aldrich, Saint-Louis, MO, US) was dissolved in 100% ethanol, and then stored at 4°C. Stock solutions were prepared at different concentrations: 20, 2000, and 6500 mg L^{-1}. For each experiment, 1 ml of stock solution was added to 1 L of water. Control groups were incubated in a 1:1000-diluted ethanol solution. For the YY feminization group treated with EE2-supplemented feed, 500 mg of EE2 was dissolved in 600 ml of 95% ethanol, and mixed with 1 kg of food pellets. It was then allowed to dry and stored at 4°C.

Early Feminization of XY Individuals

To test the sex-inversion efficiency of hormonal treatment on developing embryos, feminization treatments were applied to one of the five XY progeny batches. The treatment consisted of a single 4 hr immersion in a 2000 μg EE2 L^{-1} solution (XY2000 group) at 1 dpf, and was conducted by transferring the eggs 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 then added to the water. During incubation, the water was oxygenated by an air diffuser. After 4 hr of immersion, the eggs were netted, the solution gently removed by soaking on paper, and the eggs were rinsed in six different baths to remove hormone residues. The first three baths contained 1:1000 ethanol and the next three had only hatchery water. The batches corresponding to control groups (XYC) were handled in the same way. After rinsing, eggs were returned to the hatchery.

In order to verify that the 1 dpf feminization treatment targeted the early developmental stages before 10 days rather than by tissue accumulation of the hormone followed by a delayed effect after 10 dpf, we treated the third batch of each progeny at 10 dpf with a 4-hr immersion in a 20 μg EE2 L^{-1} solution (XY20 group). This lower dose was used to reach an EE2 tissue concentration similar to the residual concentration measured in the XY2000 group at 10 dpf. The immersion procedure was essentially the same as the egg treatment.

For steroid analysis, individuals were sampled in XYC, XY2000, and XY20 groups at 0 (unfertilized eggs, n = 67–100), 1 (n = 80–100), 4 (n = 70–100), 10 (n = 40–60), 11 (n = 20–40), 14 (n = 15–30), 21 (n = 6–10), 28 (n = 6–10), and 35 (n = 6–10) dpf. Samples were weighed and stored at −80°C until needed for steroid extraction.

Gene expression analysis was performed on individuals sampled at 4 (n = 50) and 14 (n = 20–50) dpf in control and XY2000 groups. Samples were preserved in RNA Later (Ambion, Austin, TX, US) at −20°C following the manufacturer's instructions, before RNA extraction.

Early Feminization of YY Individuals

Four YY progeny batches were produced and divided into three. The treated batches were immersed for 4 hr in a 6500 μg EE2 L^{-1} solution at 1 dpf (YY6500), following the same protocol as described above for the XY-treated eggs. Preliminary results (unpublished) showed that treatment with a 2000 μg EE2 L^{-1} solution had no effect on YY sex reversal, so we increased the EE2 concentration for the YY treatment in proportion to the EE2 concentrations used in the dietary-hormone treatment (150 mg kg^{-1} for XY and 500 mg kg^{-1} for YY) (unpublished data). Control batches (YYC) were immersed in a solution containing only ethanol (1:1000). The third group (YYD) was fed dietary hormone, and this batch served as a positive control for sex reversal. The fry from the YYD batch were fed a EE2-supplemented diet (500 mg kg^{-1} food) ad libitum from 10–40 dpf to verify the susceptibility of each progeny to the feminization treatment applied during the known sensitive period of sex differentiation.

Samplings for steroid analysis were carried out in YYC, YY6500, and YYD at 0 (unfertilized eggs, n = 100), 1 (n = 80–100), 4 (n = 65–150), 10 (n = 30–100), 14 (n = 15–60), 21 (n = 6–15), 28 (n = 6–10), 35 (n = 6–10), 40 (n = 6–10), and 42 (n = 6–10) dpf. Samples were weighed and stored at −80°C until needed for steroid extraction.

Gene-expression analysis was performed on individuals sampled at 4 (n = 40–50) and 14 (n = 20–50) dpf in control and YY6500 groups. Samples were preserved in RNA Later (Ambion) at −20°C, following the manufacturer's instructions, before RNA extraction.

Sex-Ratio Analysis

Phenotypic sex was determined by the acetocarmine squash method (Guerrero and Shelton, 1974) at 90 dpf. Fish were euthanized by an overdose (500 mg L^{-1}) of benzocaine (Sigma–Aldrich), and a slice of the gonads from 53–125 fish per batch was microscopically examined after acetocarmine coloration.

Steroid Extraction

Steroids were extracted from homogenized samples (0.25–1.15 g weight) following a procedure described in D'Cotta et al. (2001). After three extractions with dichloromethane, extracts were suspended in 300 μl of 100% ethanol and stored at −20°C until needed for assessment.

TABLE 2. Primers Used for the Quantitative Real-Time PCR

Gene	GenBank accession number	Forward primer	Reverse primer
cyp19a1b	AF295761	F957: 5′-GATTCATGAAGCCGAGAAGC	R1184: 5′-TTCAAGATGGTGTTCATCATCTCCT
amh	EF512167	F1577: 5′-AAGCAGCGCAAACATTAACA	R1741: 5′-GTTCCAGTCCACAACCTCCA
foxl2	AY554172	F310: 5′-AAGAGGAGCCGGTTCAGGACAA	R396: 5′-GCTCTCCCGGATAGCCATGG
ef1 α	AB075952	F832: 5′-TGTTGAGACTGGTATCCTGAAGCC	R1072: 5′-GATGATGACCTGAGCGTTGAAGC

EE2 Enzyme Immunoassay

EE2 was assayed by enzyme-linked immunosorbent assay following the manufacturer's instructions (Europroxima, The Netherlands). Twenty microliters of extract were dissolved in 180 μl of dilution buffer provided with the kit. Each sample from treated groups was further diluted to reach a concentration in the detection range (0.02–2 ng g^{-1}) of the kit. All samples were assayed in duplicate.

Testosterone, 17β-Estradiol, and 11-Ketotestosterone Radioimmunoassays

Testosterone (T), 17β-estradiol (E2), and 11-ketotestosterone (11KT) concentrations were assayed in developing eggs, larvae, and juveniles by radioimmunoassay, as described in Douxfils et al. (2007). Radioactive hormones were purchased from Amersham Pharmacia (Buckingamshire, England). T and E2 antibodies were obtained from the Laboratoire d'Hormonologie de Marloie (CER, Belgium), and the anti-11KT was a gift from A. Fostier (INRA, Rennes, France). Detection limits ranged from 50–80 pg ml^{-1}.

Total RNA Extraction and Reverse Transcription

Heads were dissected from their trunks under a stereomicroscope, and placed in RNA Later. Each batch sampled was divided into two or three replicates, and each replicate contained from 6–17 heads. Total RNA was extracted from pooled heads using TRIzol reagent (Invitrogen, Carlsbad, CA, US), following the manufacturer's protocol, after which total RNA was resuspended in 30–150 μl of RNase-free water and quantified with a NanoDrop (Thermo Scientific, Wilmington, DE, US). Extracts were treated to remove genomic DNA with TURBO DNase (Ambion), and single-strand cDNA was produced using SuperScript II reverse transcriptase (Invitrogen), as described in Poonlaphdecha et al. (2011). Reverse transcription was performed with 5 and 4 μg of total RNA for samples from 4 and 14 dpf, respectively. The cDNA was diluted in water to 25 ng μl^{-1} and stored at −20°C for quantitative real-time PCR.

Quantitative Real-Time PCR Analysis

Expression levels of *cyp19a1b*, *amh*, and *foxl2* were studied in embryo (4 dpf) and fry heads (14 dpf). Quantitative PCR was performed on a MX3000P (*cyp19a1b* and *amh*) or MX3005P (*foxl2*) real-time PCR system (Stratagene, La Jolla, CA, US). Due to its expression stability during estrogen exposure (data not published), *ef1α* was used as a reference to standardize gene expression levels. The primers used are listed in Table 2. All biological replicates were analyzed in duplicate from 10 μl reactions using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, US). Reactions were run with 50 ng cDNA and a concentration of 300 nM for each primer. PCR cycling parameters, control of reaction specificity, and calculation method for gene expression levels (expressed as mean normalized expression) are described in Poonlaphdecha et al. (2011, 2013).

Data Analysis

Data are reported as mean ± standard error of the mean. Mean values of growth, steroid concentrations, and relative gene expressions were compared between groups (treated versus control). Normality was analyzed with the Shapiro–Wilk test, and homoscedasticity with the Levene test. If data complied with these two conditions, differences between means were searched by ANOVA, and multiple comparisons were performed with the Fisher's LSD test. Otherwise, nonparametric Kruskal–Wallis test and Mann–Whitney test were used. The chi-squared test was used to analyze survival and sex reversal rates. Spearman rank correlation coefficients were used to identify correlations between sex reversal rates, steroid concentrations in unfertilized eggs, and changes in gene expression levels. Differences were considered as significant at $P < 0.05$. Statistical analysis was performed using Statistica v.10 (StatSoft, France).

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