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RESEARCH ARTICLE

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Do sex reversal procedures differentially affect agonistic behaviors and sex steroid levels depending on the sexual genotype in Nile tilapia?

Vincent Gennotte[1] | Balagizi Akonkwa[2] | Charles Mélard[1] | Mathieu Denoël[3] | Charlotte A. Cornil[4] | Carole Rougeot[1]

[1]Aquaculture Research and Education Center (CEFRA), University of Liège, Tihange, Belgium

[2]Laboratory of Hydrobiology, Official University of Bukavu, Bukavu, D. R. Congo

[3]Laboratory of Fish and Amphibian Ethology, Behavioural Biology Unit, Freshwater and Oceanic Science Unit of Research (FOCUS), University of Liège, Liège, Belgium

[4]Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA) Neurosciences, Research Group in Behavioral Neuroendocrinology, University of Liège, Liège, Belgium

Correspondence
Vincent Gennotte, Aquaculture Research and Education Center (CEFRA), University of Liège, Chemin de la Justice 10, B-4500 Tihange, Belgium.
Email: vgennotte@ulg.ac.be.

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Abstract

In Nile tilapia *Oreochromis niloticus*, phenotypic males and females with different sexual genotypes (XX, XY, YY) have particular behavioral and physiological traits. Compared to natural XX females and XY males, XY and YY females and XX males expressed higher level of aggressiveness that could be related to higher levels of 17β-estradiol and 11-ketotestosterone, respectively. Our results suggest that the presence of a Y chromosome increases aggressiveness in females. However, since the same relationship between aggressiveness and the Y chromosome is not observed in males, we can hypothesize that the differences in aggressiveness are not directly dependent on the genotype but on the sex reversal procedures applied on young fry during their sexual differentiation to produce these breeders. These hormonal treatments could have permanently modified the development of the brain and consequently influenced the behavior of adults independently of their genotype. In both hypotheses (genotype or sex reversal influence), the causes of behavioral modifications have to be searched in an early modification of the brain sexual differentiation.

1 | INTRODUCTION

Developmental processes leading to a sexually differentiated organism are noteworthy in fish. From the diversity of their sex determination systems to the lability of their gonad and brain sexual differentiation, the ontogenetic steps of organization of sex differences in fish are singular among vertebrates (Devlin & Nagahama, 2002). In mammals, the gonad phenotype is genetically determined early during development and gonadal hormones subsequently shape irreversible sex differences in the brain that modulate physiology and behaviors in adulthood (MacLusky & Naftolin, 1981). In teleosts, the genetic and endocrine dialog between the developing brain and gonad in the process of sexual differentiation is not fully deciphered but the developmental polarity of these structures could be reversed compared to mammals, the differentiated brain determining the fate of the gonads. This idea was first proposed by Francis (1992) and is now supported by increasing evidence regarding sex dimorphic gene expression and steroid metabolism in the brain (Blázquez & Somoza, 2010; Diotel et al., 2010; Godwin, 2010). The reversed developmental polarity (i.e. sexual differentiation of the brain prior to the gonads) may be an adaptive mechanism that provides high plasticity of sexual differentiation and the bipotentiality characterizing both gonads and brain (Godwin, 2010; Munakata & Kobayashi, 2010). This concept is well figured in hermaphroditic species in which external factors such as change in social structure can trigger a total phenotypic sex change, with adapted behaviors related to the sexual phenotype (Devlin & Nagahama, 2002). Observations made on hermaphrodites suggest that both male and female characteristics are present in the brain of fish and can be adaptively activated in response to their physiology or environmental influences (Munakata & Kobayashi, 2010). The plasticity of sex

differentiation is not restricted to hermaphrodites as exemplified by the numerous sex reversal experiments performed in diverse gonochoristic species. Sex reversal procedures usually rely on the administration of sex steroids or the exposure to environmental factors such as temperature that override the genetic sex determinism (Baroiller & D'Cotta, 2001; Pandian & Sheela, 1995). Even in gonochoristic fish, the sexual plasticity could extend throughout adulthood as suggested by Paul-Prasanth et al. (2013) who reported the sexual inversion of XX adult medaka (*Oryzias latipes*) and Nile tilapia (*Oreochromis niloticus*) by long-term estrogen depletion. In this experiment, sex-reversed XX fish expressed male-typical behavior and mated with females, showing that the brain sexual bipotentiality is conserved in adulthood.

In *O. niloticus*, sex reversal procedures allow to produce all the combinations of sexual phenotypes and genotypes (XX, XY and YY males and females). These fish are used in aquaculture for the control of reproduction and the improvement of growth by all-male mass production (Baroiller & Jalabert, 1989; Toguyeni et al., 2002). Fish with atypical combinations of sex chromosomes and sexual phenotype are important tools for studying the mechanisms of sex determination and the influence of the genotype on biological traits such as reproductive performances and behavior.

In the African pygmy mouse (*Mus minutoides*), one of the rare mammals harboring an unusual sex determination system with XX and XY females, these latter compensate some loss of fitness by improved reproductive performances, leading to the production of more offspring compared to XX females (Saunders et al., 2014). Differences in behavior, and especially in aggressiveness level, were also described in XY female laboratory mice (*M. musculus*) (Gatewood et al., 2006). In reptiles, Li, Holleley, Elphick, Georges, and Shine (2016) reported differences in morphology, physiology, and behavior between concordant ZW females and sex-reversed ZZ females in the Central Bearded Dragon, *Pogona vitticeps*. In fish, comparison of physiological and reproductive features like sex steroid profiles and sperm quality showed no difference between XX sex-reversed and XY normal males in salmonids (Fitzpatrick, Henry, Liley, & Devlin, 2005; Geffen & Evans, 2000) and European perch (*Perca fluviatilis*) (Rougeot et al., 2004). Similarly, we observed comparable sperm characteristics in XX, XY, and YY males in *O. niloticus* (Gennotte et al., 2012). On the contrary, particular biological traits were reported in sex-reversed females of blue tilapia (*O. aureus*), a closely related species with a ZZ/ZW sex determination system. In this species, when ZZ females competed with ZW females for reproduction, the spawning rate of ZZ females was lower (Desprez & Mélard, 1998) and related to a higher level of dominance and aggressiveness than in ZW females (Ovidio, Desprez, Mélard, & Poncin, 2002). Desprez and Mélard (1998) hypothesized that the phenotypic sex reversal could be incomplete, particularly in the brain, and that the interaction of genotypic and endocrine factors could modify the expression of behavior.

Hormones, especially sex steroids, are important actors of the expression of social behaviors, including aggressiveness. Differences in profiles of circulating steroids depend on the sexual phenotype but also on the social status, dominant and more aggressive fish generally exhibiting higher androgen levels than subordinates (O'Connell, Ding, & Hofmann, 2013; Oliveira, Hirschenhauser, Carneiro, & Canario, 2002). However, relationships between hormones and behavior are complex and hormones are considered as behavioral modulators rather than the trigger of agonistic behavior (Oliveira & Gonçalves, 2008).

The aim of this study was to assess the influence of sexual genotype (XX, XY, and YY) and the role of circulating sex steroids on the expression of agonistic behaviors in *O. niloticus*. Threatening and fighting behaviors were quantified in pairs involving a male and a female with various sexual genotype combinations. Basal levels of testosterone, 17β-estradiol, and 11-ketotestosterone were measured in a standardized context avoiding influence of reproduction and sexual arousal. These data provide new insights on the influence of sex chromosomes in the process of brain sexual differentiation and on the possible role of sex steroids in translating genotypic differences to the behavioral level.

2 | MATERIALS AND METHODS

2.1 | Fish

Nile tilapias from the Lake Manzala strain were from the Research and Education Center in Aquaculture (CEFRA), University of Liège, Belgium. Six groups of fish with different combinations of sexual phenotype and genotype were tested: XX males (*XXm; n = 22), XY males (XYm; n = 34), YY males (YYm; n = 22), XX females (XXf; n = 34), XY females (*XYf; n = 22), and YY females (*YYf; n = 22). Sex-reversed fish, characterized by a sexual genotype opposite to their phenotype, are annotated with an asterisk (*) to distinguish them from fish with concordant sexual genotype and phenotype, which were not exposed to a hormonal sex reversal treatment. All-male XX progenies were obtained by mating XX males with normal XX females and feeding fry with 65 mg·kg^{-1} diet of 17α-methyltestosterone (MT) (Sigma-Aldrich, Saint-Louis, MO, US) during the first 30 days of feeding (from 10 days postfertilization). YY males were obtained by mating YY males with YY females. YY females resulted from the same cross followed by phenotypic sex reversal by alimentary administration of 17α-ethynylestradiol (EE2) (Sigma-Aldrich, Saint-Louis, MO, US; 500 mg·kg^{-1}) during the first 30 days of feeding. XY individuals produced by mating YY males with XX females were sex-reversed by a 30-day administration of EE2 (Sigma-Aldrich, Saint-Louis, MO, US; 150 mg·kg^{-1}) to obtain XY females. XY males and XX females came from normal cross between XY males and XX females. Experiments were conducted on 18-month-old fish. Individual weight was standardized in order to avoid bias in the establishment of dominance and agonistic interactions. Mean body weights (±SE) were as follows: *XXm: 274 ± 8 g, XYm: 276 ± 8 g, YYm: 279 ± 6 g, XXf: 227 ± 4 g, *XYf: 225 ± 5 g, *YYf: 230 ± 7 g. Mean body weights, compared by analysis of variance (ANOVA), were statistically similar among female groups (ANOVA: $F_{2,98} = 0.177$, $P > 0.05$) and among male groups (ANOVA: $F_{2,95} = 0.099$, $P > 0.05$). Sex-reversed individuals were phenotypically indistinguishable from normal ones. Male and female phenotypes were distinguished based on the genital papilla morphology (Popma & Masser, 1999). All individuals had a functional sexual apparatus: males were able to emit milt and females were able to emit oocytes by strip-

ping. Before the experiment, fish were stocked in 300-L tanks (density: 50 kg·m^{-3}) in a recirculating system at 27–28°C with a 14-hr light/10-hr darkness photoperiodic regime. Feeding was performed once a day at satiation with commercial tilapia diet (38 % proteins, 7 % lipids; Coppens, Helmond, the Netherlands).

Animal care and the experimental protocol were approved by the local ethic committee of the University of Liège and were conducted in compliance with the European legislation on animal welfare and experimentation.

2.2 | Behavioral records and analysis

Agonistic behaviors were observed in fights staged between one male and one female. Five different crosses of broodstock fish with different sexual genotypes were tested: XYm × XXf, XYm × *XYf, XYm × *YYf, *XXm × XXf, and YYm × XXf. In each cross, one of the two breeders had a normal sexual genotype (XYm or XXf). The pairs XYm × XXf were considered as controls. Each confrontation was repeated with six different pairs of fish. In each confrontation, the male displayed a body weight 20 % higher than the female in order to respect the natural sexual dimorphism in Nile tilapia (Toguyeni et al., 1997).

Prior to observation, fish were acclimated in 250-L aquaria in a flow-through system (temperature: 27°C; photoperiod: 14-hr light/10-hr darkness). Each aquarium was split into two compartments by an opaque partition and accommodated two individuals. Male was on the side of the water inlet and female on the side of the water outlet. During the acclimatization period, fish were fed four times a day with special broodstock commercial tilapia diet (45 % proteins, 5 % lipids; Coppens, Helmond, the Netherlands). In order to standardize their physiological state, acclimatization was adjusted on the ovarian maturation cycle of the female. After the first spawn of the female, unfertilized eggs were gently removed from her mouth and behavior recording was performed two days after egg removal in order to avoid influence of handling stress. As the mean duration of a maturation cycle is 15 days in a non-mouthbrooding female of *O. niloticus* (Tacon, Baroiller, Le Bail, Prunet, & Jalabert, 2000), courtship behaviors were not expressed at the beginning (on the third day) of a cycle and observations focused on agonistic behaviors.

Behaviors were recorded with a video camera (webcam Pro 9000, Logitech, Lausanne, Switzerland) connected to a computer and placed behind a screen to prevent any visual contact between the fish and the experimenter. Each pair of fish was video-recorded four times over the day. Sequences were filmed at 0900, 1100, 1300, and 1500 hr. Fish were not fed on the day of observation. Fish were staged by removing the opaque partition 10 min before the first recording. Because aggressive interactions were more intense just after pairing, the first sequence was longer and lasted 10 min; the following recordings were run for 5 min (total recording time: 25 min).

Behavioral analysis was performed using The Observer XT software (Noldus Information Technology, Wageningen, the Netherlands). Threats and attacks were classified as agonistic behaviors, which are easily distinguishable from courtship behaviors. A detailed description of these behaviors was made by Baerends and Baerends-van Roon (1950) and Falter (1983) and summarized by Longrie et al. (2013) (Table 1). Quantification was performed by counting behaviors expressed as punctual events in terms of frequencies (hr^{-1}). On the other hand, behaviors defined as a sustained state were quantified by measurement of duration and expressed as a proportion (%) of the total recording duration. In this case, the sum of the three concerned behaviors can exceed 100 % as these behaviors can be concomitant.

TABLE 1 Description and quantification method of the agonistic behaviors observed in *O. niloticus* confrontations (one male vs. one female), adapted from Baerends and Baerends-van Roon (1950), Falter (1983) and Longrie et al. (2013)

Behavior	Measurement	Description
Threats		
Fin raising	Duration	Dorsal, caudal, and anal fins are erected
Throat swelling	Duration	Buccal cavity puffs up by spreading of the branchiostegal membrane; generally accompanied and emphasized by opercular spreading.
Chasing	Duration	Rapid swim toward the opponent.
Frontal display	Count	Frontal approach of the opponent, usually accompanied by a throat swelling and opercular spreading.
Attacks		
Lateral attack	Count	The attacker violently puts his mouth on the side of the opponent; can ends by a bite.
Tail beating	Count	A sudden or repetitive slap(s) of the tail; fish are side by side.
Mouth fighting	Count	Fish are face-to-face and press their wide open mouth one against the other.
Biting	Count	Usually on the side or fins.

2.3 | Steroid assay

Blood was sampled in XX, XY, and YY males and females to measure serum concentration of sex steroids. Fish were transferred from stock tanks to the same facility and held in the same conditions as described above for behavioral records (250-L aquaria split in two compartments). Aquaria were first loaded with males, then with females. Sixteen individuals from each sexual phenotype/genotype combination were sampled. Fish were selected in the same stocks as for behavioral observations but were different individuals. Males were acclimatized 1 week in aquarium before sampling. Acclimatizing period of females was adjusted on their maturation cycle as described above. Females were sampled two days after their first spawn. Fish were anaesthetized (benzocaine, Sigma-Aldrich, Saint-Louis, MO, US; 40 mg L^{-1}) and blood was sampled by caudal venipuncture. After centrifugation (4,500 rpm, 20 min, 10°C), serum was stored at −20°C until analyses.

Serum concentrations of testosterone (T), 17β-estradiol (E2), and 11-ketotestosterone (11 KT) were assayed by radioimmunoassay after two extractions with cyclohexane-ethyl acetate (1:1) as described in Douxfils et al. (2007). All samples and standards were assayed in duplicate. Radioactive hormones were purchased from Amersham Pharmacia (Buckingamshire, England), the T and E2 antibodies from the Laboratoire d'Hormonologie , CER Groupe (Marloie, Belgium), and the anti-11KT was provided by Dr. A. Fostier (INRA, Rennes, France). Intra-assay coefficients of variation were 4.2, 6.1, and 3.5 % for T, E2, and 11KT, respectively. Detection limits ranged from 50 to 80 pg mL^{-1}.

2.4 | Data analysis

Expressions of behaviors were separately compared between males and between females. As behavioral data sets did not comply with both homoscedasticity and normality, even after relevant data transforming, analyses were performed using the nonparametric Kruskal–Wallis (KW) test, followed by appropriate multiple comparisons of mean ranks (Siegel & Castellan, 1988). Concentrations of circulating sex steroids were compared between the six groups (XX, XY, YY males and females together). Normality and homoscedasticity of hormonal data sets were first improved by application of Box Cox transformation. Transformed data were compared by ANOVA followed by post hoc Newman–Keuls test for multiple comparisons. Differences were considered as significant at $P < 0.05$. Behavioral data are graphically represented using box plots showing the median, the 25 and 75% percentiles, the minimum, and maximum. Results are presented in different boxes for males and females, except for the mouth fighting behavior that involved the two partners at an identical level. Statistical analysis was performed using Statistica 13 (StatSoft, Tulsa, OK, US).

3 | RESULTS

3.1 | Agonistic behaviors

Fin raising (Fig. 1a) was expressed from 0 to 47% time in pairs with an XXf and from 29 to 76% time in pairs with a *XYf or *YYf. *YYf displayed a significantly higher median expression level (62% time) than control XXf (9–11% time) ($P = 0.015$). Among XYm, those confronted to *XYf and *YYf erected their fins at a similar level to females, significantly higher ($P < 0.019$) than the control XYm. Within crosses with XXf, *XXm showed an intermediate level (27% time) of fin raising not significantly different from the other males.

Throat swelling (Fig. 1b) median expression level was significantly higher in *YYf (48% time; $P = 0.006$) than in control XXf (8% time). In males, throat swelling was significantly ($P = 0.041$) more expressed in XYm (52% time) paired with *YYf than in control XYm (12% time) paired with XXf. Throat swelling median expression level was also high in XYm confronted to *XYf (46% time) but statistically not different ($P > 0.05$) from control XYm. *XXm, XYm, and YYm paired with XXf expressed throat swelling at a similar low level (11–16 % time; $P > 0.05$).

Chasing (Fig. 1c) was observed at a very low level in crosses *XXm × XXf, YYm × XXf, and XYm × XXf (median level ranged from 0 to 2% time) and was more frequent in males and females in XYm × *XYf (median: 43 and 33% time, respectively) and XYm ×*YYf (median: 12 and 13%, respectively). The time spent in chasing was very variable in these two crosses, depending on the pairs observed (from 0 to 58% time). KW test revealed a significant difference among female groups ($P = 0.007$) but multiple comparisons were unable to identify the source of difference. No statistical difference was observed between males ($P > 0.05$).

Frontal display (Fig. 1d) was expressed in a similar pattern. The median frequency of expression of this behavior ranged from 0 to 4 hr^{-1} in confrontations with an XXf and from 2 to 20 hr^{-1} in confrontations with a *XYf or *YYf. KW test revealed a significant difference among female groups ($P = 0.047$) but multiple comparisons were unable to identify the source of difference. No statistical difference was observed between males ($P > 0.05$).

Lateral attacks (Fig. 1e) were significantly more frequent in *YYf (median: 48 hr^{-1}; $P = 0.041$) compared to XXf paired with YYm (median: 4 hr^{-1}). Frequencies of attacks were not significantly different between control XXf (median: 4 hr^{-1}) and the other groups of females. Among males, median expression level was high in XYm paired with *YYf (median: 35 hr^{-1}) and in XYm paired with *XYf (median: 23 hr^{-1}). However, observed values were variable and not statistically different form the control XYm ($P > 0.05$).

Tail beating (Fig. 1f) median expression levels ranged from 0 to 48 hr^{-1} in females and from 22 to 59 hr^{-1} in males. The XYm × *XYf confrontations showed the highest level of expression, significantly higher than the control for the males ($P = 0.017$).

Mouth fighting (Fig. 1g) expression was significantly different between groups ($P = 0.021$) but multiple comparisons failed to identify the source of difference. The highest levels of expression were observed in XYm × *XYf (median: 11 hr^{-1}) and XYm × *YYf (median: 11 hr^{-1}) confrontations compared to the control (median: 0 %).

Biting (Fig. 1h) behavior was not observed in crosses YYm × XXf and XYm × XXf and expression was low and not significantly different from 0 in *XXm × XXf ($P > 0.05$). This behavior was more frequently expressed in crosses XYm × *XYf (median: 5 hr^{-1} for both sexes) and XYm × *YYf (male median: 12 hr^{-1}, $P = 0.007$; female median: 16 hr^{-1}, $P = 0.006$).

The total time spent in three state behaviors (fin raising, throat swelling, and chasing) was significantly higher in *XYf ($P = 0.028$) and *YYf ($P = 0.016$) than in control XXf (Fig. 2a). The % time spent in these behaviors ranged from 17 to 54% (median: 41%) for *XYf, from 33 to 51% (median: 42 %) for *YYf, and from 3 to 17% (median: 8%) for all the XXf. Among males, the expression of these behaviors was significantly higher in XYm staged with *XYf (median: 44% time, XYm vs. control XYm: $P = 0.006$) and staged with *YYf (median: 39% time, XYm vs. control XYm: $P = 0.004$) than in the control XYm (median: 9% time).

The sum of the event behaviors (lateral attack, frontal display, tail beating, mouth fighting, and biting) followed a similar profile with a higher expression level in crosses XYm × *XYf and XYm × *YYf than in the other ones (Fig. 2b). The total count of these agonistic behaviors reached median values of 95 and 94 hr^{-1} in *XYf and *YYf, respectively, and 104 and 112 hr^{-1} in XYm staged with them. Agonistic behaviors were expressed at a lower frequency in crosses with XXf (median

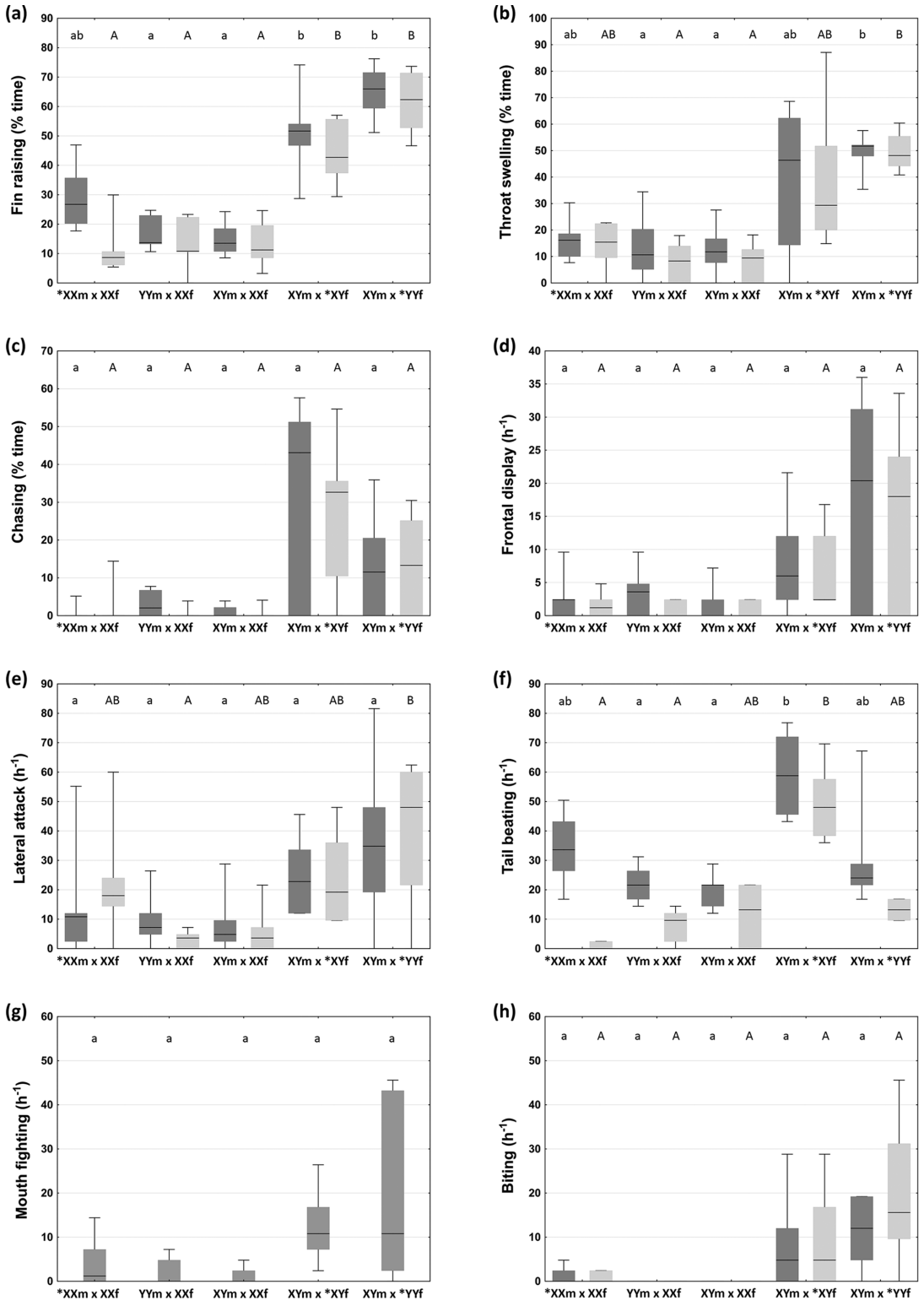

FIGURE 1 Agonistic behavior levels in five types of confrontation staged between one male and one female *O. niloticus* with different sex genotypes: *XXm × XXf, YYm × XXf, XYm × XXf (control), XYm × *XYf, and XYm × *YYf ($n = 6$). Sex-reversed fish are annotated with an asterisk (*) to distinguish them from fish with concordant sexual genotype and phenotype. Fin raising (a), throat swelling (b), and chasing (c) behaviors are expressed as % of observation time. Frontal display (d), lateral attack (e), tail beating (f), mouth fighting (g), and biting (h) behaviors are expressed as number per hour. Bar: median, box: 25 and 75 % percentiles, whiskers: minimum and maximum. Different upper case letters indicate significant differences between females, and different lower case letters indicate significant differences between males. Dark gray: males, light gray: females

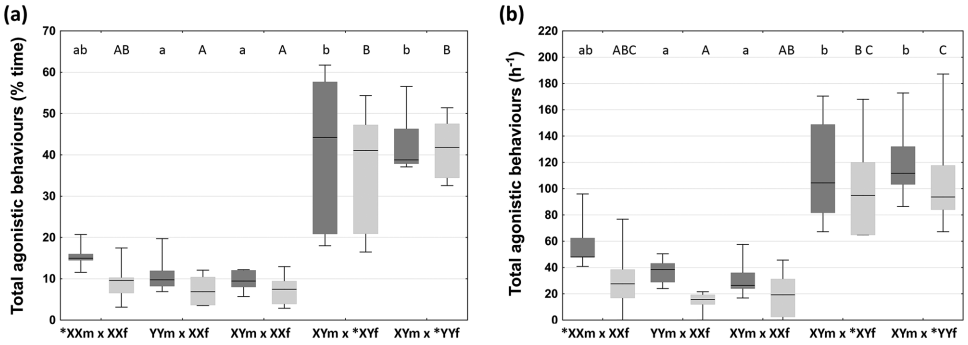

FIGURE 2 Summary of agonistic behavior levels in five types of confrontation staged between one male and one female *O. niloticus* with different sex genotypes: *XXm × XXf, YYm × XXf, XYm × XXf (control), XYm × *XYf, and XYm × *YYf ($n = 6$). Sex-reversed fish are annotated with an asterisk (*) to distinguish them from fish with concordant sexual genotype and phenotype. (a) Mean duration (% time) of state behaviors: fin raising, throat swelling and chasing. (b) Total number (hr^{-1}) of event behaviors: lateral attack, frontal display, tail beating, mouth fighting, and biting. Bar: median, box: 25 and 75% percentiles, whiskers: minimum and maximum. Different upper case letters indicate significant differences between females, and different lower case letters indicate significant differences between males. Dark gray: males, light gray: females.

ranged from 16 to 28 hr^{-1} and from 26 to 48 hr^{-1} in females and males, respectively).

3.2 | Sex steroids

Mean T serum concentration was significantly ($P < 0.001$) higher in males than in females (overall mean values: 34.7 ± 6.4 and 4.3 ± 0.6 ng mL^{-1}; overall median values: 20.4 and 3.4 ng mL^{-1}, respectively), but within the same sex phenotype, no statistical difference ($P > 0.05$) was observed between the different sex genotypes (Fig. 3a). Mean circulating level of E2 was similar in the three male genotypes (*XXm: 4.7 ± 1.1 ng mL^{-1}, XYm: 2.5 ± 0.2 ng mL^{-1}, YYm: 4.7 ± 0.8 ng mL^{-1}; $P > 0.05$) (Fig. 3b). In females, the highest concentrations of E2 were measured in the YY genotype. E2 level in *YYf (mean: 14.2 ± 2.2 ng ml^{-1}) was significantly different ($P = 0.001$) from the concentration in XXf (mean: 6.5 ± 1.4 ng mL^{-1}). *XYf was characterized by an intermediate level (mean: 9.5 ± 1.7 ng mL^{-1}). No difference ($P > 0.05$) in mean 11KT concentrations was observed between XXf, *XYf, and *YYf (XXf: 3.8 ± 0.5 ng mL^{-1}, *XYf: 3.4 ± 0.5 ng mL^{-1}, *YYf: 2.9 ± 0.2 ng mL^{-1}) (Fig. 3c). Higher levels were measured in males with a concentration in *XXm (mean: 26.5 ± 4.2 ng mL^{-1}) significantly higher than in XYm (mean: 16.0 ± 4.1 ng mL^{-1}, $P = 0.019$) and YY (mean: 16.4 ± 2.8 ng mL^{-1}, $P = 0.032$).

4 | DISCUSSION

The most striking result of the present study was the higher expression of agonistic behaviors in *XYf and *YYf compared to XXf. From low-intensity (displays and threats) to high-intensity acts (attacks), a similar behavioral expression profile was observed with a higher aggressiveness in XYm × *XYf and XYm × *YYf confrontations. Although this conclusion can be nuanced when considering behaviors individually, the global pattern emerging from all agonistic behaviors together gives an unequivocal view of the aggressiveness level in these individuals. The higher aggressiveness level in these pairs can be attributed to the female since the intensity of fights was far lower in pairs with normal genotypes XYm × XXf. In every type of confrontation, the male slightly expressed more agonistic behaviors than the female, according to its larger size and the social and territorial behavior of this species (Baerends & Baerends-van Roon, 1950; Boscolo, Morais, & Gonçalves-de-Freitas, 2011). *Oreochromis niloticus* has a lek mating system in which male defends a territory to ensure his reproductive success. But even out of a reproductive context, a hierarchical social structure sets up inside a group in which dominant individuals fervently defend a territory against intrusion (Baerends & Baerends-van Roon, 1950). If the intruder is not much aggressive, as the XXf, the interaction rapidly finds an outcome in which the dominant establishes its status against the subordinate. On the contrary, if the intruder is more combative (or more responsive to its opponent's aggressions) and fights to win the confrontation, an aggressive escalation is initiated for the establishment of dominance (Boscolo et al., 2011; Carvalho & Gonçalves-de-Freitas, 2008; Oliveira, Carneiro, & Canario, 2005). To avoid this experimental drawback (one opponent responds to the other at the same level), results should be refined by performing male–male and female–female confrontations.

Regarding our main results, two hypotheses can be proposed to explain the higher aggressiveness observed in females carrying Y chromosome. The first one is a direct influence of sexual chromosomes on behavior through brain sexual differentiation, or on activation of neural mechanisms related to aggressiveness expression, as proposed by Desprez and Mélard (1998) and Ovidio et al. (2002) to explain the higher aggressiveness level observed in sex-reversed ZZ females compared to ZW females in *O. aureus*. Brain organizational or activational modifications would be under control of genetic factors linked to the Y chromosome or induced by the absence of a twin copy of factors carried on the X chromosome. This kind of chromosomal influence on sex differentiation is observed in sex reversal experiments. The required dose of EE2 to feminize YY individuals by dietary administration is more than three times higher than the efficient dose for XY (see Materials and Methods section, unpublished data). Likewise, high-temperature treatment (36°C) applied during the sensitive period for sex differentiation induces different effects in XX (masculinization), XY (none), and YY (feminization) (Kwon, McAndrew, & Penman, 2002). Thermal and hormonal sex reversals evoked here could rely on their

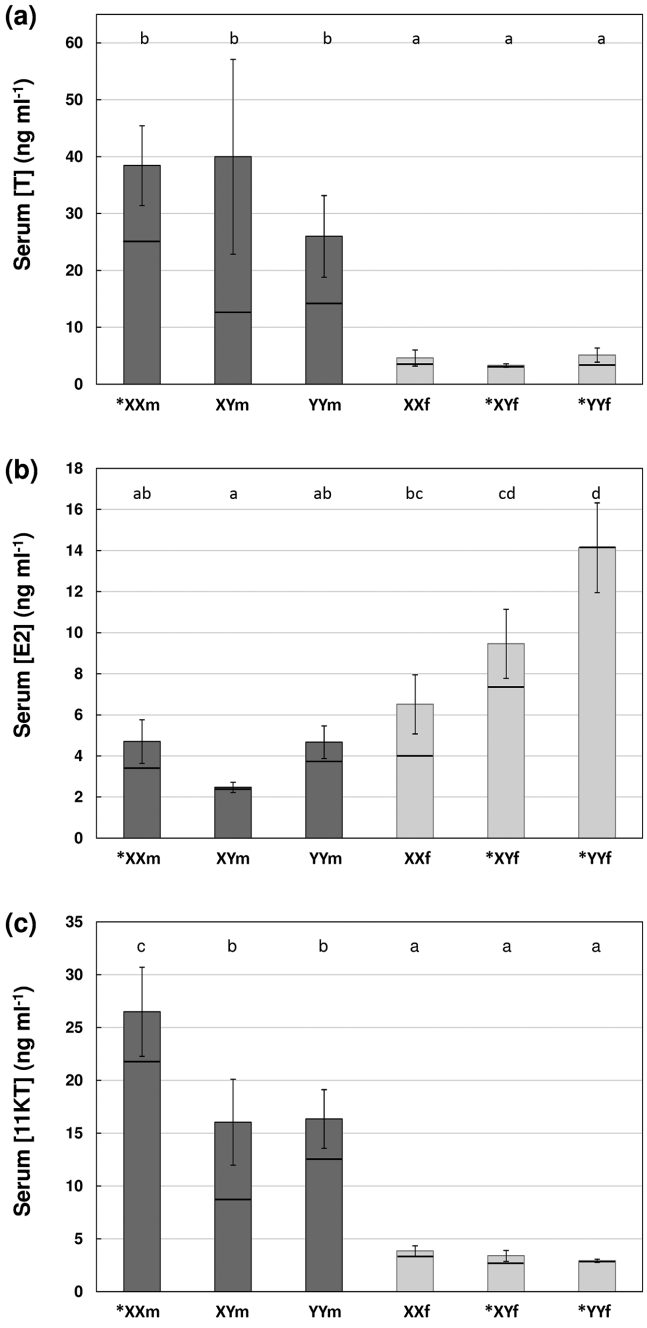

FIGURE 3 Circulating levels (mean ± SE, horizontal black lines represent median values) of (a) testosterone (T), (b) 17β-oestadiol (E2), and (c) 11-ketotestosterone (11KT) in adult male and female *O. niloticus* with different sex genotypes: XX, XY and YY. Sex-reversed fish are annotated with an asterisk (*) to distinguish them from fish with concordant sexual genotype and phenotype. Different letters indicate significant differences. Dark gray: males, light gray: females

own mechanisms, different from that involved in normal brain and behavior differentiation, but all of them imply that genetic determinants carried by sexual chromosomes in zero, one, or two copies can modify the sexual plasticity and differentiation in response to specific stimuli.

In mice, XY females produced by decoupling the major sex determinant *Sry* from the sexual genotype expressed higher aggressiveness compared to XX females. These results showed that aggressive behaviors are influenced by the presence of both *Sry* and other sex-linked genes carried on sex chromosomes that are expressed even in the absence of the major sex determinant of testis development (Gatewood et al., 2006). Contrary to mammals, sex chromosomes of *O. niloticus* and other fishes are at an early evolutionary stage of differentiation (Volff, Nanda, Schmid, & Schartl, 2007), but we can assume that such genes linked to the sex chromosomes may influence different aspects of sex differentiation, specially the behavior. A relative autonomy of sex-linked genetic factors, associated with a brain sexual differentiation prior to the gonad (Godwin, 2010; Munakata & Kobayashi, 2010) could provide to fish their exceptional sexual plasticity through a decoupling of the different components of sex phenotypical differentiation (gonad, brain, behavior). This plasticity is demonstrated in hermaphroditic species with social determinism and species with alternative male phenotypes (Almeida, Canario, & Oliveira, 2014; Godwin, 2010). Even if adults of *O. niloticus* with different sexual genotypes are sexually functional, producing fertile gametes and expressing mating and spawning behaviors (Billy & Liley, 1985; Gennotte et al., 2012), the decoupling of components controlling sex expression and the expression of sex-linked genetic factors could explain a genotypic modulation of behavioral aspects like aggressiveness.

Nevertheless, the hypothesis of an increase in aggressiveness linked to the Y chromosome is not supported by the observations made on males. However, our experiment was not specifically designed to highlight differences in male aggressiveness levels. Male–male confrontations should be studied to solve this issue and definitely rule out the chromosomal influence hypothesis. In this study, XXf was less aggressive and less responsive to male behavior. Consequently, whatever the genotype, asymmetrical fighting abilities lead the male to rapidly win the confrontation and express few agonistic behaviors.

A second hypothesis, based on an influence of the hormonal sex reversal treatments on the brain sexual differentiation, could also explain the difference in aggressiveness level observed between groups with different genotypes. Only fish with a sexual genotype opposite to their phenotype (*XYf and *YYf), and exposed to a sex reversal treatment during the sensitive period of sex differentiation, displayed modified levels of aggressiveness compared to controls (XYm and XXf). The case of *XXm was uncertain as these fish were also sex-reversed but a marked difference in aggressiveness level was not reported with this experimental design. Global expression values of *XXm were higher than in control XYm but not statistically different. Behavioral effects of steroids as endocrine disrupting chemicals at environmental relevant concentrations are documented (Söffker & Tyler, 2012) but few data exist on the organizational effects of sex reversal treatments at early developmental stages. Androgens and estrogens influence many aspects of brain development and differentiation (Cooke, Hegstrom, Villeneuve, & Breedlove, 1998). In particular, estrogens play a crucial role in neurogenesis regulation as well as brain sexual differentiation and expression of brain aromatase expression is upregulated by estrogens and aromatizable androgens (Diotel et al., 2010). Consequently, the heavy supply of EE2 (to *XYf and *YYf) and perhaps MT (to *XXm) during the sensitive period of sex differentiation could have permanently affected the development of the brain.

Tsai and collaborators (Tsai & Wang, 1998, 1999; Tsai, Wang, Chang, & Kao, 2000; Wang & Tsai, 1999) reported an influence of both E2 and MT, administered in similar sex reversal treatments in Mozambique tilapia (*O. mossambicus*), on the production of several neurotransmitters (noradrenaline, γ-aminobutyric acid, glutamate, and serotonin), suggesting that these neurotransmission pathways could be involved in brain sexual differentiation or reflect the produced sex difference. In mammals, these neurotransmitters are involved in the development of sexual dimorphism in brain structure and function (Wilson & Davies, 2007) and play regulatory roles in aggressiveness expression (Haller, 2013). Less data exist in fish but serotonin is also known to modulate aggressive behaviors (Summers et al., 2005; Winberg & Nilsson, 1993). Moreover, Billy and Liley (1985) reported an increase in aggressiveness level in adult Mozambique tilapias exposed to a masculinizing treatment of MT during the sensitive period of sex differentiation. Behavioral modifications were not only observed in sex-reversed males (*XX), but also in genetic males (XY) submitted to the same hormonal treatment, suggesting that hormonal treatments administered during the sex differentiation period influence behavioral differentiation. Interestingly, another kind of environmental influence acting on the first ontogenetic stages and showing long-term influence in adults was reported in reptiles. In the leopard gecko (*Eublepharis macularius*), differences in reproductive and agonistic behaviors as well as sex steroid levels were observed between individuals of the same phenotype but incubated at different temperature at the embryonic stage (Gutzke & Crews, 1988).

The second question addressed in this study concerned the influence of sexual genotype on the level of circulating sex steroids and their potential regulatory role in agonistic behavior expression. In fish, the neural pathways underlying aggressive behavior expression are modulated by androgens (Oliveira et al., 2002; Oliveira & Gonçalves, 2008). Consequently, even though an increased agonistic motivation of *XXm compared to XYm and YYm has to be confirmed, this could be linked to a higher level of 11KT. Alteration of the steroid metabolism could have been induced by the hormonal treatment used for sex reversal rather than by a genetic influence of sex chromosomes as no difference in 11KT level was observed between XYm and YYm and between female groups. 11KT is synthesized in testes but not in the brain as supported by the absence of 11β-hydroxylase in rainbow trout (*Oncorhynchus mykiss*) (Liu et al., 2000) and zebrafish (*Danio rerio*) (Diotel et al., 2011). However, the production of gonad steroid is under control of the hypothalamo–pituitary complex by the release of gonadotropins, and the gonadotropic function is itself controlled by diverse neuropeptides and neurotransmitters and by a steroid negative feedback (Zohar, Munoz-Cueto, Elizur, & Kah, 2010). Developmental alteration of these regulatory pathways could therefore modify the androgen production by the gonads.

This mechanism is also valid for estrogens. In our study, E2 level increased in females carrying a Y chromosome (from XXf to *YYf) but this trend was not observed in males. Similarly to 11KT, sex reversal treatments—made with higher dose of EE2 in *YYf than in *XYf—could have permanently modified estrogen synthesis. Moreover, unlike 11KT, E2 is produced in the gonads and in the brain. Brain aromatase activity is high in fish and correlated to circulating sex steroid levels (Diotel et al., 2010) supporting the idea that differences in E2 level can have a cerebral origin. Disruption of the ontogeny of the gonadotropic system and the expression of brain aromatase was reported in *D. rerio* after exposure to EE2, this steroid inducing an increase in the number of gonadotropin-releasing hormone immunoreactive neurons and a modification of their migration profile as well as an induction of brain aromatase expression (Vosges et al., 2010).

However, beside a differential development of the brain, we have to consider a possible influence of the reproductive cycle on the E2 levels in females. It is not known if oocyte maturation speed is the same in *XY and *YY females, as physiological aspects like growth can be affected by the sexual genotype (Toguyeni et al., 2002). In our study, behavioral measurements and blood sampling were performed on the second day of the maturation cycle and mean E2 levels in XX females increase from 5 to 12 ng mL^{-1} during the first 3 days of a maturation cycle (Tacon et al., 2000), which is in the range of our measurements in the three different genotypes.

The role of estrogens in the expression of agonistic behaviors is less documented than the role of androgens and results are sometimes divergent. In birds and mammals, administration of E2 can have a facilitating role in the expression of aggressiveness (Laredo, Villalon Landeros, & Trainor, 2014; Soma et al., 2000). In fish, administration of exogenous estrogens inhibits aggressiveness in different species (Colman, Baldwin, Johnson, & Scholz, 2009; Saaristo, Craft, Lehtonen, & Lindström, 2010). However, in *Astatotilapia burtoni*, an African cichlid fish, Huffman, O'Connell, and Hofmann (2013) reported that aromatase activity and high level of E2 promote aggressive behaviors in males, suggesting that a relationship can exist between the higher aggressiveness and the E2 levels in *XY and *YY female *O. niloticus*.

In conclusion, in *O. niloticus* either sex chromosomes or hormonal sex reversal treatments may have an organizational influence on brain sex differentiation resulting in a differential expression of agonistic behaviors in adulthood. These organizational effects may also have altered sex steroid levels that can modulate aggressiveness expression and could have wider biological influences, particularly on reproductive behavior. Even if some evidence (asymmetrical effect of sex chromosomes in males and females, and behavioral modification in sex-reversed fish only) provide more support in favor of a hormonal explanation of the origin of behavioral alteration compared to genotype, the question remains open and in both cases, the cause has to be searched in an early modification of brain sexual differentiation. To disentangle the two hypotheses, different types of confrontation (e.g. male–male) should be tested and all genotypic variants with a same sexual phenotype should be exposed to an identical hormonal treatment during development (e.g. 65 mg MT kg^{-1} for *XXm, XYm, and YYm; 500 mg EE2 kg^{-1} for XXf, *XYf, and *YYf) in order to standardize the effect of sex reversal procedures among the different genotypes.

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