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Sex-ratio, early sex steroid profiles and *cyp19a1b*, *dmrt1* and *foxl2* gene expressions upon high temperature treatment of undifferentiated African catfish juveniles (*Clarias gariepinus*)

Saïdou Santi[a,b,*], Vincent Gennotte[a], Marc Muller[c], Charles Melard[a], Aboubacar Toguyeni[b], Syaghalirwa N.M. Mandiki[d], Carole Rougeot[a]

[a] Aquaculture Research and Education Center (CEFRA), Liège University. 10 Chemin de la Justice, B-4500 Tihange, Belgium
[b] Aquaculture and Aquatic Biodiversity Research Unit/Natural Resources and Environmental Sciences Research and Studies Laboratory (UR-ABAQ/LERNSE), Institute of Rural Development (IDR), Nazi Boni University, BP 1091, Bobo-Dioulasso, Burkina Faso
[c] Laboratory for Organogenesis and Regeneration (LOR), GIGA-Research, Liège University, Avenue de l'Hôpital, B-4000 Liège, Belgium
[d] Research Unit of Environmental and Evolutionary Biology (URBE), Institute of Life, Earth and Environment (ILEE), Namur University, Rue de Bruxelles 61, B-5000 Namur, Belgium

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ABSTRACT

Different mechanisms are involved in high temperature-induced masculinization among teleost fishes. These mechanisms need to be investigated in African catfish, a species with a XX/XY genetic sex determinism that can be overridden by high temperature (HT). The influence of HT on the three main sex steroid hormones (testosterone – T, 17β-estradiol – E2, and 11-ketotestosterone – 11KT) was assessed from non-fertilized eggs to 14 days post-hatching (dph) in eggs or whole body of six full-sib progenies reared at 28 °C (control) and 36 °C for 6 to 8 dph (high masculinizing temperature). HT effect on *foxl2*, *cyp19a1b* and *dmrt1* expression was also investigated in the head at the end of the thermal treatment (8 dph) and shortly after (14 dph) in the six progenies. Masculinization rates in fish exposed to HT ranged from 30 to 100%, but did not influence sex steroid levels. However, the masculinization rate was correlated with 11KT levels at 1-h post-fertilization, suggesting that 11KT level at this phase may influence the masculinization potential. Expression of *foxl2* was not detected in the head during the experimental period while, to our knowledge, this is the first report of *dmrt1* expression in teleost fish head (perhaps in brain). Similar to sex steroid hormones, HT did not induce significant changes in *cyp19a1b* or *dmrt1* relative expressions in the brain. Nevertheless, when results were split into two groups, according to the masculinization rates of progenies, lower thermosensitive progenies showed high *cyp19a1b* relative expression shortly after HT treatment, while in control groups, highly thermosensitive progenies displayed high *dmrt1* relative expression at 14 dph. While this may be true, a direct link between the HT treatment and the biological indicators analyzed was not clear and the mechanisms of masculinization have to be further investigated, particularly in the gonad.

1. Introduction

African catfish, *Clarias gariepinus*, is a species with a XX/XY genetic sex determination system (Galbusera et al., 2000) influenced by temperature (Santi et al., 2016). Although a large body of literature focused on the mechanisms through which temperature affects sex differentiation in fish, like the Nile tilapia (which share the same biotope with African catfish) (Baroiller and D'Cotta, 2016; Baroiller et al., 2009; Baroiller et al., 1999; Shen and Wang, 2014), no data exists concerning African catfish, to our knowledge.

The classical view of sex development in fish supposed that sex-determining genes (major factor) act as upstream genes controlling downstream genes, that are referred as sex-differentiating genes, and which in turn control sex steroid hormone production to promote the appropriate sexual phenotype. The new concept suggests that sex is not defined through a single genetic cascade or hierarchical cascade within a genetic network, but through a continuum in which genetic, parental, environmental and random factors act together in a non-hierarchical

* Corresponding author at: Aquaculture and Aquatic Biodiversity Research Unit/Natural Resources and Environmental Sciences Research and Studies Laboratory (UR-ABAQ/LERNSE), Institute of Rural Development (IDR), Nazi Boni University, BP 1091, Bobo-Dioulasso, Burkina Faso
E-mail address: saidou.santi@gmail.com (S. Santi).

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network to influence cell proliferation and hormone levels and consequently induce the appropriate sexual phenotype (Baroiller and D'Cotta, 2016; Heule et al., 2014; Mei and Gui, 2015; Perrin, 2016). Temperature acts on sex steroid hormone levels through the regulation of sex-linked gene expression or on germ cell proliferation through epigenetic or genetic processes (Baroiller and D'Cotta, 2016; Piferrer, 2013; Selim et al., 2009; Uchida et al., 2004).

The 17β-estradiol (E2) is main sex steroid influencing sex differentiation in fish. Its upregulation and downregulation induced respectively female and male differentiation (Diotel et al., 2010; Tokarz et al., 2015). E2 synthesis from testosterone or androstenedione is catalyzed by the cytochrome P450 aromatase, the transcription product of *cyp19a1* gene (D'Cotta et al., 2001b; Guiguen et al., 2010). In fish, two isoforms of aromatase encoded by two different genes with different expression patterns were reported: the gonadal aromatase gene (*cyp19a1a*) and the brain aromatase gene (*cyp19a1b*) (Diotel et al., 2010; Kwon et al., 2001; Tchoudakova and Callard, 1998). The ovarian differentiation pathway is triggered and maintained by upregulation of gonadal aromatase, whereas *cyp19a1a* is downregulated in testicular differentiation (Guiguen et al., 2010; Piferrer et al., 2012). The repression of aromatase gene expression by HT is suggested to contribute to masculinization in thermosensitive species (Baroiller et al., 2009; Guiguen et al., 2010; Piferrer et al., 2012). Indeed, D'Cotta et al. (2001b) showed (in Nile tilapia) that aromatase gene expression was repressed during HT treatment; in both genetic male and masculinized female gonads. These authors suggested that aromatase gene repression is required in gonads (and perhaps in the brain) to drive sex differentiation towards testis development. *Foxl2* (forkhead box protein L2), which activates aromatase transcription, was inhibited by HT in both Nile tilapia and Japanese flounder (*Paralichthys olivaceus*) (Baroiller et al., 2009), consistent with the decreased *cyp19a1a* gene expression.

Nonetheless, HT-induced masculinization is not only the result of female pathway repression, but can result from upregulation of the male differentiation pathway. In Nile tilapia, HT first induced an increase of *dmrt1* (doublesex and Mab-3 (DM) related transcription factor 1) and *amh* (Anti-müllerian hormone) gene expressions in gonads of temperature-masculinized females (XX), followed by repression of *foxl2* and *cyp19a1a* gene expressions, thus overriding the chromosomic female sex (Poonlaphdecha et al., 2013). In this species, HT also induced an upregulation of other testicular differentiating genes such as *sox9a*, *sox9b*, *dax1* and *cyp11b* (involved in 11-ketotestosterone – 11KT biosynthesis) (Baroiller and D'Cotta, 2016). Similarly, a raise in 11KT level induced by HT was observed in pejerrey (*Odontesthes hatcheri*) (Hattori et al., 2009). In medaka, HT treatment during embryonic stages induced a high expression of the *dmrt1* gene in genetic females, suggesting that HT accelerates the molecular pathway leading to testicular differentiation (Hattori et al., 2007).

In teleosts, aromatase expression is high in the brain compared to mammals, resulting in high production of neuroestrogens (Blazquez and Somoza, 2010; Le Page et al., 2010). The brains aromatase *cyp19a1b* gene is specifically regulated there, suggesting early sexualization of the brain (Gennotte et al., 2017; Senthilkumaran et al., 2015). Its expression is more elevated in HT-induced male pejerrey (Diotel et al., 2010). Likewise, in rainbow trout (*Oncorhynchus mykiss*), brain aromatase gene expression and its enzyme activity were higher in male brain before testicular morphological differentiation (Vizziano-Cantonnet et al., 2011). These findings suggest that brain aromatase and neuroestrogens might be implicated in male brain differentiation.

The thermosensitive period for sex differentiation in African catfish occurs very early, from hatching to 23 days post-hatching (dph). Equally important, the highly thermosensitive period is short and extends from 6 to 8 dph, long before the appearance of the first histological signs of sex differentiation at 25 dph. When applied during this period, high temperature frequently masculinized progenies up to 80–100% (Santi et al., 2017, 2016). We focused the present study on the period before (0 hpf) until shortly after (14 dpf) the highly thermosensitive period, because in African catfish, some progenies were not thermosensitive after 14 dph (Santi et al., 2016). Considering that sex steroid hormones are involved in sex differentiation process and that steroidogenesis occurs during the early life stages in teleosts, we first aimed to determine the effect of HT on sex steroid hormone levels in whole body African catfish. Based on the previous reports on early expression of brain aromatase in several fish species (Devlin and Nagahama, 2002; Diotel et al., 2010; Le Page et al., 2010), we further focused on the effect of HT on *cyp19a1b* gene expression in heads, as well as some additional putative sex-differentiating genes (*dmrt1*, and *foxl2*), during and shortly after the highly thermosensitive period.

2. Materials and methods

The experiments were carried out according to the European animal welfare recommendations and to the guidelines of the University of Liège Ethical Committee (CEFRA, LA 1610488).

2.1. Fish and rearing conditions

Six full-sib progenies were obtained by artificial reproduction of domesticated African catfish breeders following the method previously described (Santi et al., 2016). Two-day post-hatching (dph) larvae were transferred into 50-l tanks at an initial stocking density of 20 larvae l^{-1}. Tanks were supplied with water from a recirculating system at 28.0 °C and exposed to a 12 L: 12D photoperiod. Fish rearing conditions were the same as described by Santi et al. (2016).

Each progeny was split into 4 batches, 2 duplicates in the treated group and 2 duplicates in the control group. In control groups, fish were reared at 28.0 ± 1.0 °C from 2 to 70 dph (until sex-ratio analysis). The treatment consisted of the application of high rearing temperature (36.0 ± 0.2 °C) from 6 to 8 dph (Santi et al., 2016). The experimental temperature was maintained in each tank with an individual heating system (Biotherm 2000 thermostat connected to two 300-W heaters). At 6 dph, the experimental temperature (36 °C) was reached within 3 h for a 3-day treatment. At the end of the treatment, water was cooled down to 28 °C within 3 h. Before and after the thermal treatment, fish were reared at 28 °C until sex-ratio analysis at 70 dph.

2.2. Sex-ratio analysis

At 70 dph, 100 fish (mean body weight: 48.11 ± 20.42 g) per batch were randomly sampled and euthanized with an overdose (200 mg l^{-1}) of benzocaïne (Sigma-Aldrich). Phenotypic sex was determined using the aceto-carmine squash method for sexing juvenile fish described by Guerrero and Shelton (1974). Sex was identified according the presence of oocytes in female gonads and on the lobular morphology of testis for males.

2.3. Sex steroid hormones assay

2.3.1. Egg, larva and juvenile sampling

Sex steroid hormone (testosterone – T, 17β-estradiol – E2, and 11-ketotestosterone – 11KT) concentrations were assessed during the thermosensitive period of sex differentiation, from non-fertilized eggs (maternal transmission) to 14 dph. One gram of eggs was sampled after spawning and at one-hour post-fertilization (hpf). Thereafter, one gram of larvae or juveniles was sampled in duplicate at 2, 4, 6, 7, 8, 10 and 14 dph. At each sampling time, ± 0.5 g of eggs were counted and 60 fish were weighed (± 0.01 mg) to determine the number of eggs and fish per sample (Table 1). All samples were stored at −80 °C before sex steroid extraction. Sampling dates were divided in three periods according to HT treatment: before (2 to 6 dph); during (7 to 8 dph) and after (10 to 14 dph).

Table 1
Number and weight (mean of 6 progenies ± SE) of eggs, larvae and juveniles *Clarias gariepinus* sampled at different ages for sex steroid hormones assay.

Age	Control			High temperature treated		
	Total used eggs/fish	MW (mg)	Extract quantities (g)	Total used fish	MW (mg)	Extract quantities (g)
NFE	3785	1.59 ± 0.10	1.005 ± 0.003			
1 HPF	2523	2.39 ± 0.15	1.009 ± 0.013			
2 DPH	1792	3.62 ± 1.24	1.006 ± 0.003			
4 DPH	1190	5.75 ± 2.10	1.007 ± 0.002			
6 DPH	1020	6.34 ± 1.93	0929 ± 0.104			
7 DPH	887	7.88 ± 3.30	0.975 ± 0.066	900	6.28 ± 2.13	0.949 ± 0.082
8 DPH	725	8.78 ± 1.97	0.949 ± 0.131	846	7.68 ± 2.05	0.940 ± 0.142
10 DPH	575	12.18 ± 4.58	1.009 ± 0.010	730	9.65 ± 3.57	0.966 ± 0.116
14 DPH	359	21.33 ± 10.65	1.008 ± 0.005	427	17.29 ± 7,15	0.981 ± 0.047

MW: mean weight; NFE: non-fertilized eggs; HPF: hour post-hatching; DPH: day post-hatching.

Table 2
List of primers used for RT-PCR and the Genbank accession number of cDNA sequences for *Clarias gariepinus* and *Clarias batrachus*.

Gene	Genbank Reference	Forward primer (5′ to 3′ direction)	Reverse primer (5′ to 3′ direction)
Cyp19a1b	GU220076.1	ACTGGATGAACCGGACTTTG	GCACCAGCATGAAGAACAGA
Foxl2	HQ680981.1	GAAGCCGCCGTACTCTTATG	CCAGTAGTTCCCCTTCCTCTC
Dmrt1	AF439561.1	TGCACACCAGTGAACCTCTC	GTATGCTGGATAGCGGGAAG
Ef1α	AB916539.1	CTCTCAGGGCTATGCTCCAG	TTCAGGTTCTTGGGGTTGTC
β-actin	JN806115.1	ACCGAAGTCCATCACAATACCAGT	GAGCTGCGTGTTGCCCCTGAG
Gapdh	AF323693.1	CTAAGCGCGTGGTAGTCTCC	GGGAGCCAAGCAGTTAGTTG

2.3.2. Sex steroid hormone extraction and assays

Sex steroid hormones were extracted from whole eggs or bodies, using the alcohol-dichloromethane method from Rougeot et al. (2007), adapted from D'Cotta et al. (2001a). The extraction efficiency was 86% for T and 11KT, and 81% for E2. After extraction, T, E2 and 11KT concentrations were assayed in these extract samples by radio-immunoassay (RIA) following the method of Fostier and Jalabert (1986) adapted by Mandiki et al. (2005). One set of assays was done for each hormone and the intra-assay coefficients of variation were 7.72%, 7.20% and 9.35% respectively for T, E2 and 11KT. The detection limits of the assays ranged from 8.56 to 9.93 pg ml^{-1} for the three steroids.

2.4. Gene expression analysis

2.4.1. Juvenile sampling

For gene expression analysis, fish were sampled in duplicate at 6, 8 and 14 dph in control and HT groups. At each sampling date, 30 fish were sampled in each replicated group (control and HT), kept in RNA Later (Thermo Fisher Scientific®) and stored at −20 °C until total RNA extraction.

2.4.2. Total RNA extraction and reverse transcription

RNA was extracted from heads and dissections were performed in RNA Later. Each sample was then divided into two replicates (15 heads each) and total RNA was extracted from each 15-pooled heads. RNeasy® Plus Universal Mini Kit of QIAGEN was used for total RNA extraction following the kit protocol. Extracted total RNA was then suspended in 80 µl of RNase-free water, quantified with NanoDrop and stored at −80 °C.

Reverse transcription (RT-PCR) was performed using the iScriptTM cDNA Synthesis Kit from BIO-RAD. One µg of each total RNA sample was transcribed to 20 µl of cDNA (50 ng $µl^{-1}$) following the manufacturer's protocol. cDNA samples were 2-fold diluted, with DNase- and RNase-free water, to obtain a final concentration of 25 ng $µl^{-1}$ and used for gene expression quantification by real-time PCR. Negative control on reverse transcription (without reverse transcriptase: – RT-) was performed for each sample.

2.4.3. Relative quantification of cyp19a1b, foxl2, and dmrt1 expression levels

Doublesex- and Mab-3-related transcription factor-1 (*dmrt1*), brain aromatase (*cyp19a1b*) and fork head transcription factor (*foxl2*) expression analysis was performed on an ABI Prism® 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster, CA, USA). Expression levels of these genes were assessed relatively to a reference gene (elongation factor 1-alpha – *ef1α*) and to control samples (6 dph). Three genes, including β-actin and glyceraldehyde-3-phosphate dehydrogenase – gapdh from *C. gariepinus* and *ef1α* from *C. batrachus*, were tested as reference genes. Only *ef1α* was used as reference gene due to its relative constant expression within our samples. *Ef1α* primers were designed from *C. batrachus*, a species close to *C. gariepinus*, because this gene sequence was unknown in *C. gariepinus*. qPCR primers were designed for each gene cDNA sequence (Table 2).

For qPCR, samples (cDNA and RT-) were amplified in triplicate within a total reaction volume of 15 µl containing 7.5 µl of MasterMix (2× TakyonTM RoxSYBR® MasterMix dTTP Blue, Eurogentec), 4.5 µl of primers (300 nM), 2 µl of cDNA (50 ng) and 1 µl of RNase- and DNase-free water. PCR conditions were as follows: 50 °C for 2 min (carry over prevention); 95.0 °C for 10 min (enzyme activation); 40 cycles of 95.0 °C for 15 s (denaturation) and 60.0 °C for 30 s (annealing / extension); and a dissociation at 95.0 °C for 15 s, 60.0 °C for 15 s and 95.0 °C for 15 s.

The relative expression of each target gene is presented as fold-change in target gene expression relative to the reference gene (*ef1α*) and to the control sample using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. Statistical analysis

Sex-ratio of the progenies was analyzed using the 2 × 2 contingency chi-square (χ^2) test. For each progeny, sex-ratio of the control group was compared against a 50:50 theoretical sex-ratio. Sex-ratio of HT group was compared against its own control (28 °C). For sex steroid hormone concentrations and relative gene expressions ($n = 6$ progenies), data normality was tested using D'Agostino & Pearson omnibus normality test before comparing means by ANOVA and multiple comparison with Sidak test. All differences were considered significant

Table 3
Sex ratio and masculinization rate (%) of *Clarias gariepinus* juveniles (70 days post-hatching) reared at 28 °C (control) or transiently exposed to 36 °C from 6 to 8 dph (HT-treated).

Progeny	Control	HT-treated	
	% Males	% Males	% Masculinization
1	48.0	64.0*	30.8
2	56.0	82.0*	59.1
3	50.0	80.0*	60.0
4	41.0	95.0*	91.5
5	41.0	97.0*	94.9
6	61.0	100*	100

* Significantly different ($p < .05$) from the control.

when $p < .05$; statistical tests and graph were performed with GraphPad Prism v. 6.00 (GraphPad Software, La Jolla California USA). The Pearson (parametric test) or Spearman (non-parametric test) correlation test was performed, whether data have respectively normal distribution or not, to determine different correlations. Correlations were considered significant when $p < .05$.

3. Results

3.1. Sex-ratio analysis

At 70 dph, the sex-ratio of control groups in all progenies was not significantly different from a theoretical 50:50 sex-ratio. Male proportion in these groups ranged from 41 to 61%. All HT-treated groups displayed a significantly ($p < .05$) skewed sex-ratio towards the male phenotype when compared to their respective controls. Male proportion in HT groups ranged from 64 to 100% with masculinization rates ranging from 30 to 100% (Table 3). To analyze steroid level and gene expression results, we divided progenies into two groups (of three progenies each) depending on their thermosensitivity and hence their masculinization rates: the first group (M60) had masculinization rates ranging from 30 to 60%, the second one (M100) from 91 to 100%.

3.2. Sex steroid hormone concentrations

Concentrations of E2 and T in non-fertilized eggs revealed an important variability between progenies. The highest concentrations were observed in non-fertilized eggs for E2 and T (14 ± 5 and $10 \pm 4\,ng\,g^{-1}$ respectively) and at 4 dph for 11KT ($2.51 \pm 0.70\,ng\,g^{-1}$). E2 and T concentrations tended to decrease during the remaining experimental period (Fig. 1). Only E2 concentrations in non-fertilized and in 1 hpf eggs of control groups were significantly different ($p < .05$) from the other developmental stages. Compared to E2 and T, 11KT levels remained stable below $3\,ng\,g^{-1}$ for the entire experimental period in both control and HT groups. In HT groups, there was no significant difference in all sex-steroid hormone concentrations from 7 to 14 dph. Similarly, no significant difference was observed between control and HT groups for T, E2 and 11KT (Fig. 1). Ratios between the concentrations of the three analyzed steroids (E2/T, E2/11KT and 11KT/T) were calculated (data not shown) but revealed no noteworthy trend or significant difference between control and HT groups.

3.3. Relation between masculinization rates and sex steroid hormone levels

To reveal possibly more subtle relations between thermal sensitivity and hormone levels, we first compared the low sensitivity M60 to the high sensitivity M100 groups at the different stages; as expected, no significant differences were observed for the three sex steroids within the control groups (Fig. 2). In contrast, the T concentration was significantly higher in M60 than in M100 HT batches from 7 to 8 dph, corresponding to the HT treatment period (Fig. 2).

In a second approach, we investigated possible correlations between sex reversal rates and sex steroid levels in individual groups. In 1-hpf eggs, 11KT concentration increased significantly ($R^2 = 0.6542$, $p = .0014$) with masculinization rates (Fig. 3). Correlations between masculinization rate and either E2 or T concentrations in all sampling data were calculated (data not shown) but revealed no noteworthy trend.

3.4. Expression analysis of putative sex-differentiating genes

Gene expression analysis was performed on mRNAs extracted from head just before (6 dph), immediately after (8 dph) and 6 days after (14 dph) HT treatment.

No expression of *foxl2* was observed on control or HT groups. *Cyp19a1b* and *dmrt1* relative expressions were not significantly different between control and treated groups during the entire experimental period or within these groups (Fig. 4). No significant differences were observed between control M60 and M100 groups for *cyp19a1b* relative expression during the entire experimental period (Fig. 5). However, the M60 group displayed a significantly higher ($p < .05$) *cyp19a1b* relative expression than M100 groups at 14 dph in the HT batch. Conversely, the relative expression of *dmrt1* in control M100 was significantly higher ($p < .05$) than control M60 at 14 dph, whereas no significant difference was observed in HT batches (Fig. 5).

3.5. Relations between sex steroid hormone levels, masculinization rates and putative sex-differentiating gene expressions

No significant correlations were found between *cyp19a1b* and *dmrt1* relative expressions and sex steroid hormones concentrations at the end (8 dph) or shortly after (14 dph) HT treatment.

Masculinization rate was not correlated with the relative expressions of *cyp19a1b* and *dmrt1* at 8 and 14 dph. There was no correlation (in control groups) between the relative expressions of these genes and the proportion of male.

4. Discussion

African catfish displays a genetic (XX/XY) sex determining system (Galbusera et al., 2000) that can be influenced by high temperature (HT) during early life stages (Santi et al., 2016). The mechanism by which HT induces masculinization in this species needed to be explored. Therefore, we concentrated our study on the period during and shortly after the masculinization treatment and investigated, for the first time in this species, the effect of HT (36 °C from 6 to 8 dph) on the levels of the three main sex steroid hormones (testosterone – T, 17β-estradiol – E2 and 11-ketotestosterone – 11KT) and on the expression of putative sex-differentiating genes (*cyp19a1b*, *dmrt1* and *foxl2*).

Although HT treatment induced a significant masculinization in all tested progenies (masculinization rates ranging from 30 to 100%), we did not observe any differences in T, E2 or 11KT levels during the thermosensitive period; suggesting that high temperature may not act directly on sex steroid production to induce masculinization in African catfish. In the Japanese flounder, HT also did not also affect whole body T levels during gonadal differentiation (Sun et al., 2013), however, E2 levels were significantly reduced during this period (Fan et al., 2017; Sun et al., 2013). In Nile tilapia (D'Cotta et al., 2001a) and European sea bass (Socorro et al., 2007), it was suggested that 11KT concentrations were not influenced by HT treatment as the expression of *cyp11b*, the gene coding for 11β-hydroxylase, was not influenced during HT treatment. In contrast, when pejerrey larvae were reared at a high-masculinizing temperature, a rise of 11KT level in the whole body (Hattori et al., 2009) and a rise of *cyp11b* expression in presumable Leydig cells was observed during the sex determination period

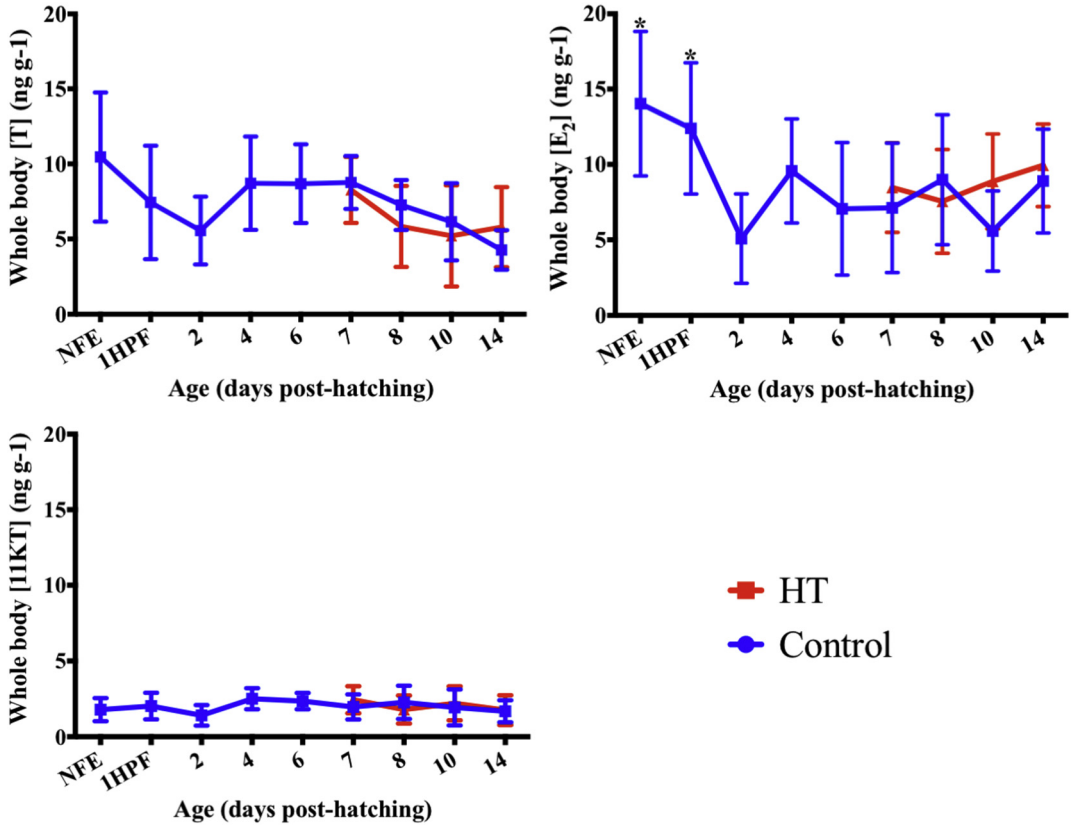

Fig. 1. Whole-body levels of testosterone (T), estradiol (E2) and 11keto-testosterone (11KT) from non-fertilized eggs to 14 dph in *Clarias gariepinus* control groups (blue) and in groups exposed to high temperature (HT, 36 °C) from 6 to 8 dph (red). Values are means ± SD of six full-sib progenies. *$p < .05$. NFE: non-fertilized eggs; 1HPF: 1-h post fertilization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fernandino et al., 2013).

In our study, high T and E2 levels were measured in non-fertilized eggs, suggesting an important transfer from maternal origin. These levels decreased during the subsequent developmental periods. Since steroids are involved in several processes such as embryonic development, metabolism, immune responses, osmoregulation or circadian rhythms (Tokarz et al., 2015), these "maternal" sex steroid hormones could be used in the brain and/or the gonads as precursor for both endogenous steroidogenesis and sex differentiation processes during early life (Rougeot et al., 2007). No correlation between the concentrations of maternal sex steroid hormones and the sex-ratio of the progenies was observed in Nile tilapia (Gennotte et al., 2014) and Eurasian perch (Rougeot et al., 2007). In contrast, in African catfish, our results reveal that 11KT levels in 1 hpf eggs are positively correlated with the masculinization rate, even though HT did not affect its production during and after treatment. Since no significant difference was observed between 11KT levels in non-fertilized and 1 hpf eggs, we assume that the 11KT levels in 1 hpf larvae result from maternal transfer. This provides preliminary evidence that there is maternal contribution to thermosensitivity of sex differentiation processes in the progenies of African catfish. Howbeit, further investigations are needed on 11KT levels in fertilized eggs during and after HT treatment to confirm this hypothesis.

The relative expression of doublesex and Mab-3 (DM) related transcription factor 1 (*dmrt1*) was measured in heads during and after HT treatment. This gene is expressed in gonads during testicular differentiation in rainbow trout, *Oncorhynchus mykiss* (Marchand et al., 2000), African catfish (Raghuveer et al., 2011; Raghuveer and Senthilkumaran, 2009), Nile tilapia (Ijiri et al., 2008) and zebrafish (Webster et al., 2017). To our knowledge, this is the first report of *dmrt1* gene expression in teleost fish head (perhaps in brain).

In Nile tilapia, *dmrt1* expression was up-regulated upon HT induction in the gonads and trunks of temperature-masculinized females (XX) (Poonlaphdecha et al., 2013), while it decreased in estrogen-treated male differentiating gonads in rainbow trout (Marchand et al., 2000). In African catfish, HT did not induce a significant change in *dmrt1* expression, neither during the experimental period nor between control and HT groups. Although no significant relations were found (in African catfish) between *dmrt1* gene relative expression and masculinization rate, neither with sex-ratio in control group, this gene showed higher expression in M100 (high masculinized progenies) than in M60 (low masculinized progenies) control group at 14 dph; signifying that the most thermosensitive progenies displayed a higher expression of *dmrt1* gene in their head (perhaps in brain) during early life stages. As *dmrt1* gene expression was assessed in the brain and it is a male-promoting gene in fish, our results suggested firstly the implication of brain in African catfish sex differentiation process and secondly that *dmrt1* could be a candidate gene for further studies on brain sexualization in teleost fishes.

Similar to *dmrt1* gene relative expression, HT did not induce significant change in either *cyp19a1b* gene expression or during the experimental period, nor between control and HT groups. In Nile tilapia, *cyp19a1b* expression did not change significantly in the brain of fish submitted to HT (36 °C) from 10 to 22 dph (Li et al., 2014), but it was down-regulated in the brain of fish submitted to HT from 10 to 19 dph. In this species, undifferentiated juveniles exhibited a sexual dimorphism of brain aromatase enzyme activity which was higher in females. HT (35 °C) induced a nearly three-fold decrease of brain aromatase activity in masculinized genetic females but also in genetic males (D'Cotta et al., 2001b). These authors also showed that HT treatment induced a repression of gonadal aromatase gene expression in the Nile tilapia. Conversely, in pejerrey, brain aromatase level is

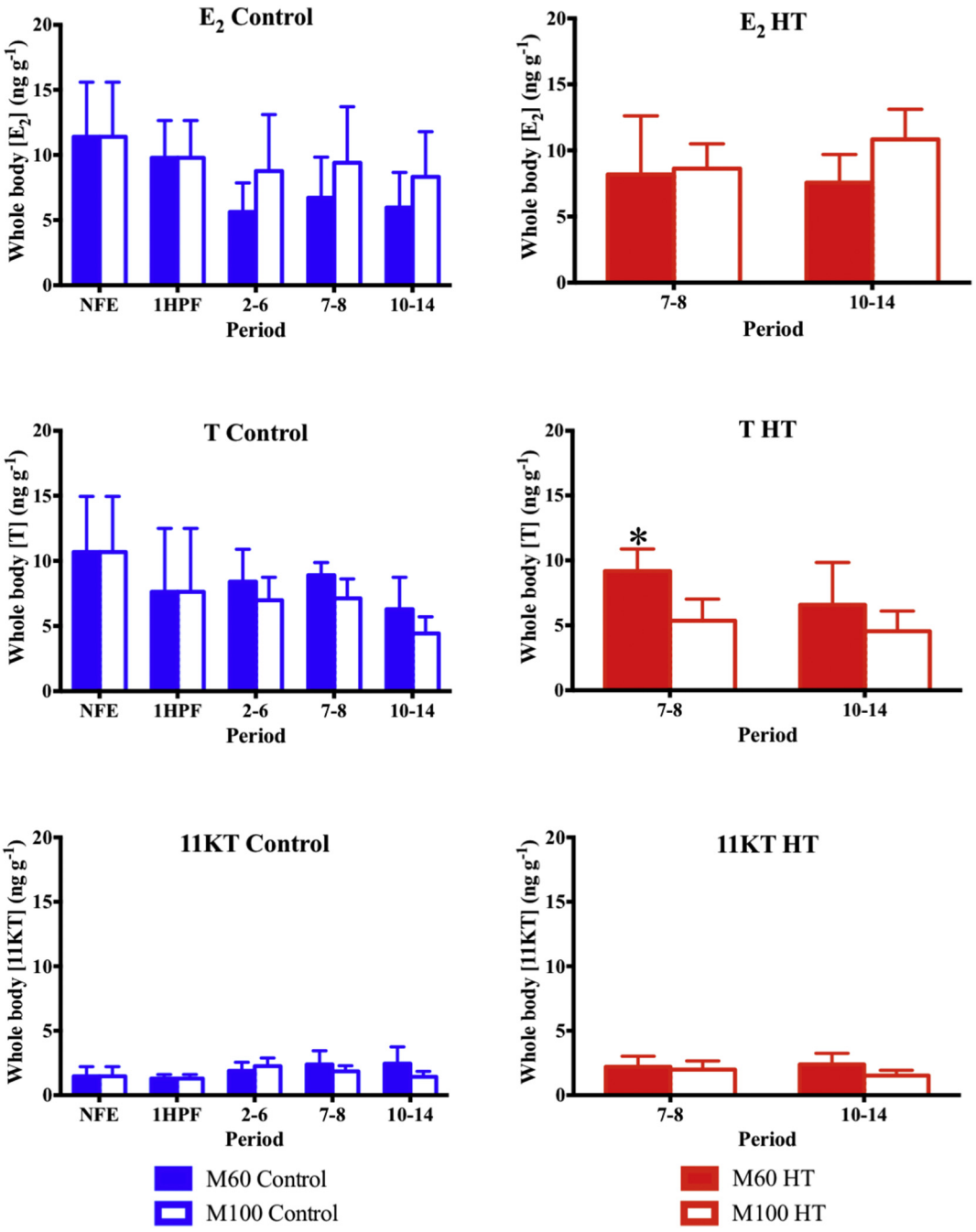

Fig. 2. Whole-body levels of testosterone (T), estradiol (E2) and 11keto-testosterone (11KT) from non-fertilized eggs to 14 dph in *Clarias gariepinus* control groups (blue) and in groups exposed to high temperature (HT, 36 °C) from 6 to 8 dph (red). Values are means ± SD of three full-sib progenies. *p < .05. NFE: non-fertilized eggs; 1HPF: 1-h post fertilization; M60: lower thermosensitive progenies; M100: higher thermosensitive progenies. M60 Low masculinized progenies (masculinization rates ranging from 30 to 60%). M100 High masculinized progenies (masculinization rates ranging from 91 to 100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

elevated in HT-promoting male (Diotel et al., 2010). *Cyp19a1b* gene was either upregulated in undifferentiated Nile tilapia treated with 17α-ethynylestradiol (Gennotte et al., 2014) or inhibited in both African catfish juveniles and Pengze crucian carp (*Carassius auratus* var. Pengze, Pcc) treated with 17α-methyltestosterone (Raghuveer et al., 2005; Zheng et al., 2016).

In the present study, no strong relations were found between *cyp19a1b* relative expression and whole body E2 concentration, neither with sex-ratio of control groups; indicating that brain aromatase did not regulate E2 concentration during the thermosensitive period in African catfish. In juveniles and adults, E2 synthesis from T is mainly catalyzed by *cyp19a1a* (gonadal aromatase) in ovaries. The high expression of *cyp19a1b* in brain is related to an auto-regulated loop of estrogen and aromatizable androgen through a mechanism that involves estrogen receptors and the estrogen response element located on the *cyp19a1b* promoter (Diotel et al., 2010; Le Page et al., 2010). Since we did not evaluate *cyp19a1a* gene expression or aromatase enzyme activity in gonad, sex steroid hormones are synthetized in several organs such as brain, gonads and kidney (Baroiller et al., 1999; Tokarz et al., 2015), it is difficult to interpret measured sex steroid hormones levels in the present study. However, when we divided the larvae into two groups according to the observed masculinization rate (M60 and M100 as

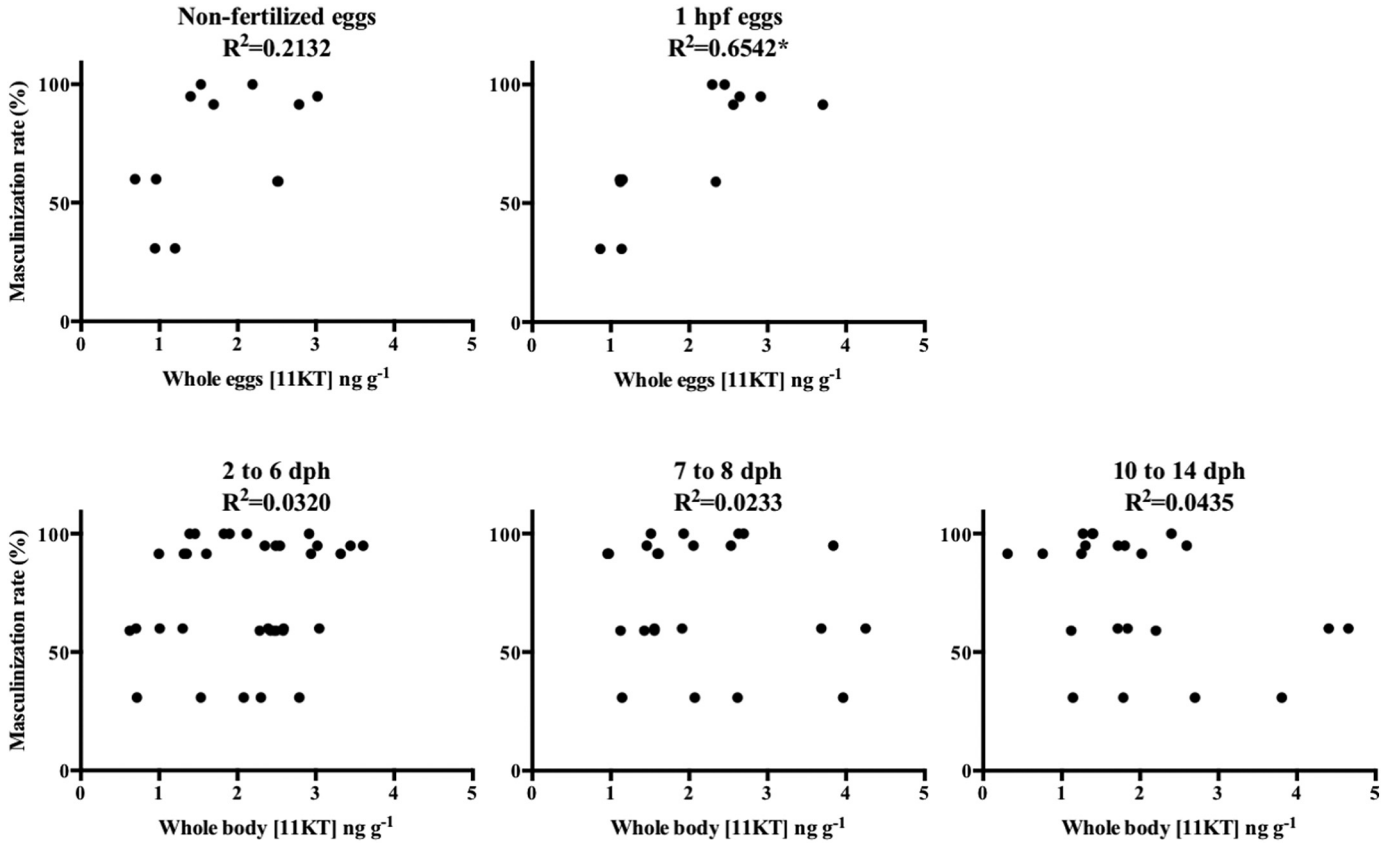

Fig. 3. Relations between masculinization rate after high temperature (36 °C) treatment (6–8 dph) and whole-body 11-keto-testosterone (11KT) levels in *Clarias gariepinus*. Each point represents duplicated value at each sampling date in the six full-sib progenies. *p < .05.

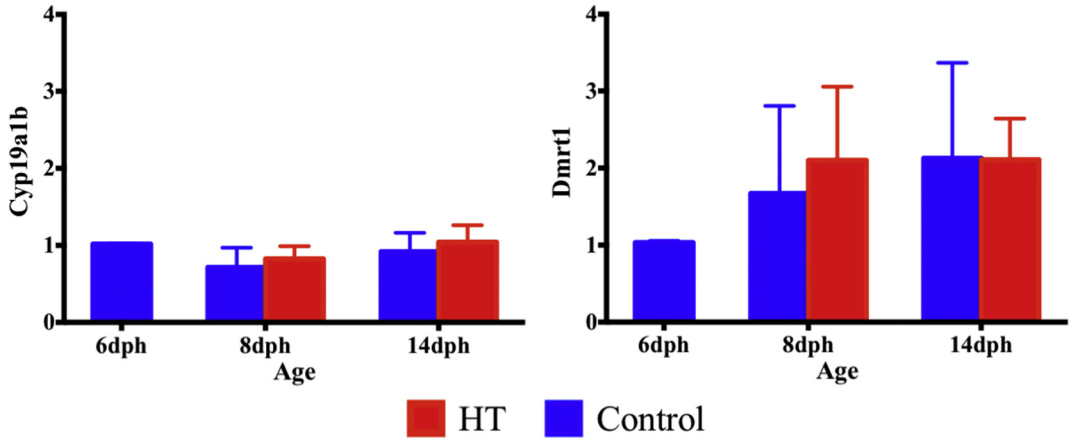

Fig. 4. Relative gene expression of *cyp19a1b* and *dmrt1* in the head of *Clarias gariepinus* control groups (blue) and high temperature (HT – 36 °C) (red)-treated groups assessed at 6, 8 and 14 dph. Bars are mean ± SD of six full-sib progenies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previously described), the group with the lowest masculinization rate displayed a significantly increased expression of *cyp19a1b* after HT treatment at 14 dph. This suggests a potential implication of brain in HT masculinizing process in African catfish.

During the experimental period, no expression of *foxl2* was measured in the heads of African catfish. Nevertheless, *foxl2* was found to regulate *cyp19a1b* expression in adult female brain (Sridevi et al., 2012). Our results suggest that the transcriptional regulation of *cyp19a1b* via *foxl2* occurs later.

This study showed that even if masculinization is induced by early exposure to HT (in African catfish), it doesn't affect the levels of whole body sex steroid hormones, nor *cyp19a1b* neither *dmrt1* expressions in the brain before histological gonad differentiation. HT temperature may act on other target (such as primordial germ cells as in others species) to induce masculinization in African catfish. Indeed, in medaka larvae, it was strongly suggested that HT induced masculinization through an elevation of whole body cortisol level, which in turn, caused an inhibition of germ cells proliferation (Hayashi et al., 2010). Similar to zebrafish, HT treatment during sex differentiation caused oocyte apoptosis, which in turn led to sex reversal of genetic females (Uchida et al., 2004). Even if histological observations of gonadal development during HT application (6 to 8 dph) were not performed in the current

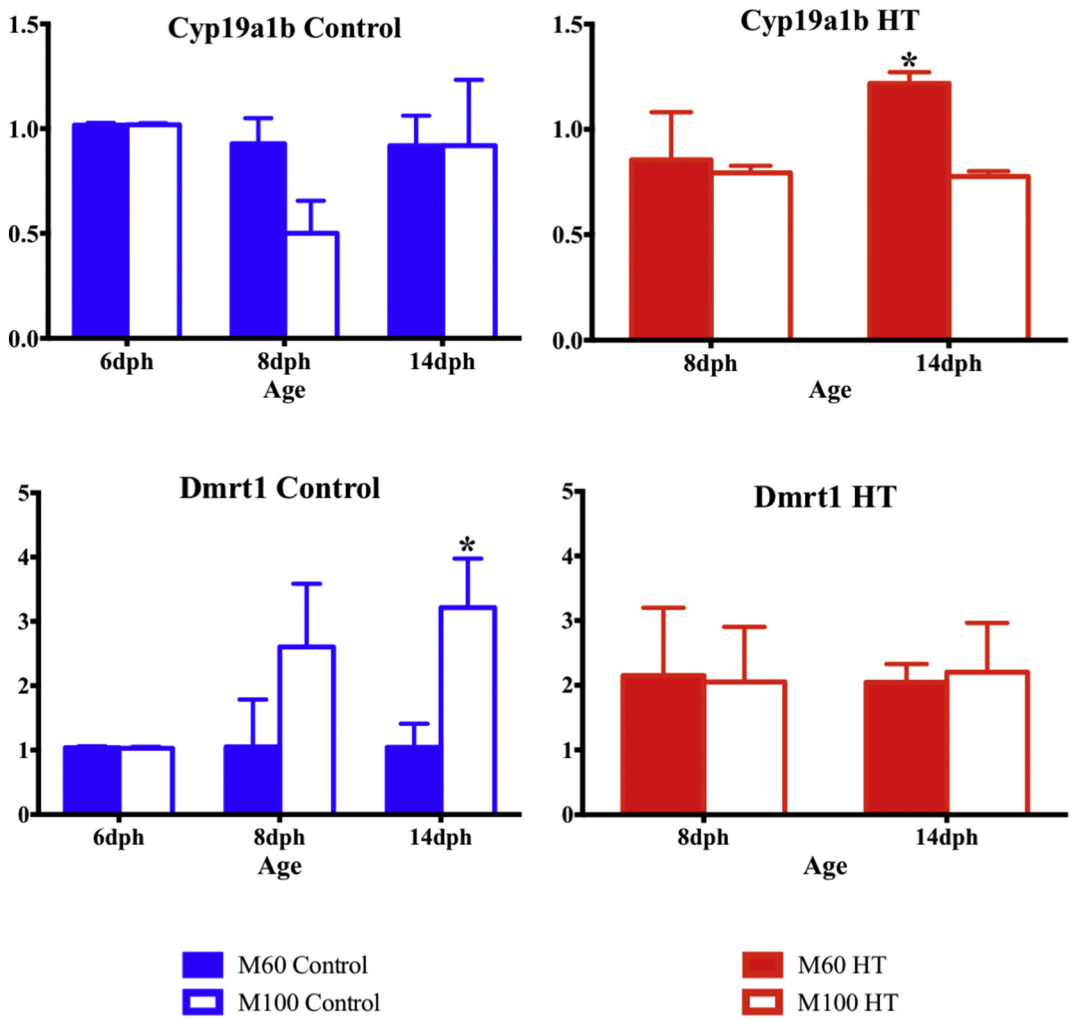

Fig. 5. Relative gene expression of *cyp19a1b* and *dmrt1* in the head of *Clarias gariepinus* control groups (blue) and high temperature temperature (HT – 36 °C)-treated groups (red) assessed at 6, 8 and 14 dph in low masculinization (30 to 60% – M60) and high masculinization (91 to 100% – M100) batches. Bars are means ± SD of three full-sib progenies. *p < .05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

study, African catfish showed undifferentiated gonads with somatic and primordial germ cells at 10 dph during normal development (Santi et al., 2016). Thus, we can hypothesize that HT treatment could influence somatic and/or germ cells to induce sex reversal in African catfish. HT could also induce a rise of cortisol concentration, a glucocorticoid stress hormone, during its application to stimulate masculinization. The role of cortisol in temperature induced sex reversal was reviewed in fish (Baroiller and D'Cotta, 2016; Fernandino et al., 2013; Shen and Wang, 2014). Regarding molecular mechanisms, HT could also influence sex differentiation through DNA methylation one of the main epigenetic process involved in fish sex differentiation (Baroiller and D'Cotta, 2016; Piferrer, 2013). Indeed, in the European sea bass and Nile tilapia, exposure to HT increased the methylation of *cyp19a* promoter in female, which was inversely correlated to gene expression in order to induce masculinization (Navarro-Martín et al., 2011; Wang et al., 2017). Therefore, investigation on the processes cited above or others in African catfish are required to elucidate HT masculinization process.

In summary, we showed that high temperature did not affect neither the levels of main sex steroid hormones (E2, 11KT and T), nor *cyp19a1b* and *dmrt1* relative expressions in the brain, but induced masculinization in African catfish progenies. Sex steroid assays have shown an important transfer from maternal origin of the three steroids that could be used as steroidogenesis in this species. Nevertheless, we demonstrated that progenies with low masculinization display a high relative expression of the *cyp19a1b* gene after high temperature treatment at 14 dph. Concomitantly, a low relative expression of the *dmrt1* gene was observed in these progenies (control group at 14 dph). These results suggest a potential implication of brain in high temperature masculinizing process in African catfish progenies.

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