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Combined effects of rearing temperature regime (thermocycle *VS.* Constant temperature) during early development and thermal treatment on Nile tilapia (*Oreochromis niloticus*) sex differentiation

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ABSTRACT

In nature, water temperature experiences daily variations known as thermocycles. Temperature is the main environmental factor that influences sex determination in most teleost fish. The purpose of this study was to examine the effects of rearing temperature (thermocycle (TC) *vs.* constant (CTE)) on development and a posterior thermal shock throughout the period of sex differentiation of Nile tilapia (*Oreochromis niloticus*). Embryos and larvae were kept under two temperature regimes: TC of 31 °C:25 °C day:night *vs.* CTE of 28 °C from 0 to 11 dpf. After this period, the larvae from each group were subjected to either heat treatment (HT, 36 °C for 12 days) or kept under the same rearing temperatures until 23 dpf (Control, C). Then all the groups remained at constant temperature until 270 dpf, when blood and gonads were collected. Larval samples were used to examine the expression of genes related to male (*amh*, *ara*, *sox9a*, *dmrt1a*) and female (*cyp19a1a*, *foxl2*, *era*) sexual differentiation. In juveniles, sex was characterized by histology, the gonadal expression of the genes involved in the sex steroid synthesis was analyzed by qPCR, and plasma testosterone (T) and estradiol (E_2) levels were analyzed by ELISA. In larvae, daily TCs increased the survival rate against HT and up-regulated the expression of ovarian differentiation genes. In juveniles, TC + C induced a higher proportion of females and higher *cyp19a1a* expression compared to CTE + C. HT induced changes in the CTE group by up-regulating testicular differentiation genes and down-regulating female promoting genes, which did not occur in the TC group. Juveniles from TC + C group presented a higher proportion of females with higher E_2 and *cyp19a1a* than CTE + HT. Fish from the CTE + HT group showed a higher percentage of males with highest T and *amh*. These findings indicate that daily TCs during larval development promote ovarian differentiation and diminish the masculinizing effects of HT.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) fish production is the second largest worldwide and is farmed in more than 80 countries, hence the keen global commercial interest and importance of this species for protein production for human consumption (FAO, 2020). Its rapid growth, good resistance to diseases, high prolificacy and early sexual maturation show great adaptability to colonize highly heterogeneous environments (Baroiller and Jalabert, 1989). Under farming conditions, however, females present a limited growth rate mainly due to their reproductive efficiency and characteristics (asynchronous ovarian development, precocious sexual maturation, mouthbrooding behavior, high energy demand for reproduction), making the management and control of mixed sexes (males and females) tilapia populations difficult. On the other hand, all-male tilapia cultures present a series of advantages such as higher growth rates and size/weight homogeneity of the population. Therefore, the tilapia aquaculture industry has made efforts to investigate sex determination in this species and mechanisms to induce masculinization of the populations destined to ongrowing and human consumption (Beardmore et al., 2001).

The Nile tilapia Sex Determination System is one of the best-documented in fish. Sex is determined by the interaction between genetic (genotypic sex determination, GSD) and environmental (environmental sex determination, ESD) factors that compose the heterogametic

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determination XX/XY system of Nile tilapia (De Alba et al., 2021b). In this species, several genes have been identified as key sex-determining genes that exhibit sexually dimorphic expression patterns before and after testis or ovary differentiation (Iriji et al., 2008; Tao et al., 2018). Tilapia ovarian differentiation is particularly encoded by factors related to *cyp19a1* (P450 aromatase enzyme), which catalyzes the conversion of androgens into estrogens. In addition, *foxl2* (*forkhead transcriptional factor L2*) is considered a key sex-determining gene of female sex differentiation in tilapia (Kobayashi et al., 2004). Testis differentiation is encoded by genes related to *doublesex- and mab-3-related transcription factor 1* (*dmrt1*) and *anti-müllerian hormone* (*amh*) (Cáceres et al., 2019; Melo et al., 2019). Dmrt1 activates the expression of *SRY-box transcription factor 9* (*sox9*) that, in turn, acts on *amh* expression (Kobayashi et al., 2008). Then Amh stimulates the production of a transforming growth factor b (TGF-b) glycoprotein that blocks *cyp19a1* expression in testes. Male sex differentiation is ensured because blocking *cyp19a1* expression prevents androgens from being converted into estrogens (Melo et al., 2019). Moreover, recent researches pointed other enzymes such as 11β-hydroxylase to play a crucial testicular differentiation (Lu et al., 2022). Ultimately, the production of estrogens like 17β-estradiol (E_2) results in female sex differentiation. Androgens like testosterone (T) and 11-ketotestosterone (11-KT) are the result of testis differentiation (Yamamoto, 1969). To complete the signaling pathway of sexual differentiation, sex steroid hormones exert their actions through estrogen or androgen receptors (Ers and Ars, respectively), which contributes to ovary or testis development, respectively (de Alba et al., 2021b).

Besides the gene control of the sex determination process in Nile tilapia, sex is also influenced by environmental conditions (ESD) (Ospina and Piferrer, 2008), of which temperature is the most pervasive factor (Baroiller et al., 2009). From very early development stages (9 dpf), undifferentiated gonads present sensitive and susceptible periods to temperature in which exposure to temperature variations can induce changes in individuals' phenotypic character by shifting the population's sex ratio (Ospina and Piferrer, 2008; Baroiller et al., 2009). However, high or low temperature treatments applied before or after sexual differentiation have been shown to have no effect on sexual development (Baroiller et al., 1995, 2009). In Nile tilapia, the critical sex differentiation period seems to range from 9 to 25 days post fertilization (dpf) (Baroiller et al., 2009). Thus high temperature treatments above 32 °C that last at least 10 days during this period can result in a higher percentage of functional male phenotypes (sex reversal by high temperature) (Kwon et al., 2000; D'Cotta et al., 2001a, b; Baroiller et al., 1995, 2009). At the molecular level, high temperatures activate both *dmrt1* and *amh by* repressing ovarian sex differentiation genes (*cyp19a* and *foxl2*) and blocking the conversion of androgens into estradiol to, thus, cause female-to-male sex reversal (Wang et al., 2017).

In the natural environment, water temperature varies through the day because solar radiation generates daily thermocycles (TCs; temperature increases during the day and drops at night) (Patterson and Wilson, 1995). In the last decade, TCs have been shown to be very relevant for several fish behavior and physiology aspects by increasing growth, feeding, thermal tolerance and survival, and by decreasing the number of malformations (Blanco Vives et al., 2011; Villamizar et al., 2012; Espirito Santo et al., 2020; de Alba et al., 2021). The effects of fluctuating temperatures on sex determination have been studied in detail in other ectothermic animals such as reptiles (Carter et al., 2018, 2019). In reptiles, fluctuating temperatures have a high impact on sex determination and this effect is mediated by the changes induced on the genes involved in this process like *foxl2*, *cyp19a1*, *drmt1* and *sox9* among others (Breitenbach et al., 2020, 2022). However less attention has been paid to date to their effects on sex determination and differentiation processes in fish (Blanco-Vives et al., 2011; Villamizar et al., 2012). In these studies, daily TCs have been reported to influence sex determination mechanisms and to modify the sex ratio in zebrafish (*Danio rerio*) and Senegalese sole (*Solea senegalensis*) with higher proportion of females in the progenies reared in TCs than at constant temperatures. In Nile tilapia, although TCs are present in their natural habitat, their importance in both sex determination and differentiation from early development stages is still unknown, as is their influence on the sex reversal process by high temperature treatment.

Taken all this previous information, our starting hypothesis was that the use of TCs would modify the process of sex determination and the response to a thermal shock during sexual differentiation in the Nile tilapia, compared with a constant temperature. This would be achieved through the influence of TCs on the molecular mechanisms involved in sex determination and differentiation and would have an impact on the final sex ratio of the different experimental groups. For this purpose, we investigated the combined effects of rearing temperature regimes (TCs *vs.* constant temperature) during early development and a posterior heat treatment (HT) during the sexual differentiation period (from 11 to 23 dpf) on the expression of genes involved in testis (*amh*, *ara*, *sox9a*, *dmrt1a*) and ovarian (*cyp19a1a*, *era*, *foxl2*) differentiation in whole larval (11 and 24 dpf) samples, plasma testosterone (T) and estradiol (E_2) levels and sex ratio in Nile tilapia juveniles.

2. Material and methods

The European Union's rules (2010/63/EU) and Spanish law (RD 53/2013, RD 1386/2018, and Law 32/2007) were followed for every procedure carried out during the experiment. The experimental protocols were approved by the regional authorities (A13220605).

2.1. Animals and housing

Male and female Nile tilapia (*O. niloticus*) juveniles (aged 1 month) were obtained from a global aquaculture company (Fishgen, Swansea, Wales, UK). Animals were placed in individual 300-L tanks that were connected to a recirculation system that had mechanical, biological, and aeration filters. A water heater and cooler (AB Aqua Medic, Gewerbepark, Germany) were used to keep the water at 28 ± 0.5 °C. Weekly measurements were made of the water quality indicators (pH, ammonia, nitrate, nitrite, and dissolved oxygen). The photoperiod was set as a 12:12 h light/dark (LD) cycle with lights on at 09:00h. The feeding regime was set using automatic feeders with three daily meals (11:00h, 15:00h and 19:00h) at a feeding rate of 2% of the total biomass per day using commercial feed (D-4 Alterna Basic 2P, Skretting, Spain) with 36% crude protein (CP).

Juveniles were reared under the previous conditions for 8 months until they reached maturity. According to their sex, tilapia individuals were separated for 2 weeks (Hussain, 2004). After this period, males and females were subjected to hormonal treatment with human Chorionic Gonadotropin hormone (hCG, Sigma Aldrich, St. Louis, USA) as described elsewhere (Fernandes et al., 2013; Espirito Santo et al., 2020). After hCG administration, fish were placed together in a male:female ratio of 3:1 for natural reproduction during the afternoon (Fernandes et al., 2013). At the beginning of the next light phase, fertilized eggs were removed from the mouth of the females. This procedure allowed us to obtain fertilized eggs with less than 12 h post fertilization (hpf), which were used in the experiments.

2.2. Experimental design

The fertilized eggs were obtained from eight different tilapia broodstocks. Four progenies and 2400 fertilized eggs (N) were used in the experiments. Each progeny came from a spawning event during which the eight broodstocks were stimulated as described above. Each progeny consisted of 600 fertilized eggs, which were obtained in the blastula stage (4–12 hpf) (Fujimura and Okada, 2007). Eggs were pooled together and distributed in incubators for Cichlid eggs (Alimar SA, Murcia, Spain) (100 eggs per incubator, six incubators per progeny used) in

two systems with different temperature regimes: a TC (thermophase:cryophase) of 31 °C:25 °C day:night *vs.* a constant temperature of 28 °C (CTE). The thermophase coincided with the light phase (09:00-21:00h) and the cryophase coincided with the dark phase (21:00-09:00h) (Fig. 1 and Suppl. Fig. A1). Electronic heaters (Askoll, Povolaro, Italy) and water coolers (Aqua Medic Titan 1500 GmbH, Bissendorf, Germany) were used to change the water's temperature at a rate of 1.5 °C per hour, mimicking the daily temperature differences that tilapia experience in the wild (Patterson and Wilson, 1995; Shoko et al., 2014; Taher et al., 2021). To ensure that thermocycles (TCs) were correctly performed and water temperature was still controlled in the CTE group, an electronic timer (Bachmann GmbH & Co, Stuttgart, Germany) was used to control temperature variations, and an underwater data logger was used to record them every 15 min (HOBO PENDANT Onset Computer Corporation, Massachusetts, USA). The photoperiod was set as a 12:12h light/dark cycle with lights on and off at 09:00h and 21:00h, respectively. Up until 7 days after fertilization, embryos and larvae were raised in incubators. Then, they were moved to 9-L tanks that were connected to the same temperature system as the larvae. At the same time (7 dpf), larvae were administered exogenous feed (Gemma, 42% CP, 0.5-0.8 μg, Skretting) as four daily meals (09:00h, 11:00h, 15:00h and 19:00h) until satiety. Larvae were reared under these temperature regimes until 11 dpf, when the survival rate was calculated as the percentage of live larvae on 11 dpf of the total number of embryos originally placed inside incubators. On 11 dpf, the larvae from TC and CTE were subjected to either HT or maintained at the same rearing temperatures regime (Control group, C) from 11 to 23 dpf (Fig. 1). HT consisted of 12 days of being exposed to 36 °C with a temperature change rate on the first and last exposure days at 1 °C/h according to the treatments followed for tilapia masculinization (Baroiller et al., 1995). At the end of HT, the survival rate was calculated as the percentage of live larvae on 24 dpf of the total number of larvae on 11 dpf. Then all groups were left at constant temperature (28 °C) until 270 dpf (Fig. 1). In addition, complete larval samples were taken on days 11 and 24 dpf (one day after HT ceased) for the mRNA expression study of the genes implicated in Nile tilapia sex differentiation and sex determination processes. According to the protocols established for the euthanasia of fish, larvae were instantly put to death by anesthetic overdose for these gene expression investigations, and their death was verified under a microscope. Then, three replicates were collected for each group or progeny, resulting in a total of 12 replicates (n = 12) from the four independent progenies. Depending on the larval stage, different numbers of larvae were employed in each replicate: two larvae/replicate on 11 dpf and one larvae/replicate on 24 dpf. Until the gene expression analysis, larvae were placed directly in 1.5 ml sterile tubes and frozen −80 °C. Finally at the end of the experiment (270 dpf), up to 25 fish from each group/progeny (up to 100 fish/group) were randomly selected and anesthetized with 50 μL/L of clove oil essence (eugenol, Guinama, Spain). In the groups of each progeny with fewer than 25 fish, all the animals from the group were used. The total number of animals sampled from each group was 92 for TC + C, 95 for TC + HT, 88 for CTE + C and 80 for CTE + HT. Before euthanasia, blood was collected by caudal puncture and whole body weight was measured. Blood was centrifuged for 15 min at 3000 rpm and 4 °C. After centrifugation, plasma was separated and frozen at −80 °C until analyzed. In addition, one lobule of the gonads from each fish were collected, transferred to sterile 1.5 ml RNase- and DNase-free Eppendorf tubes which were frozen rapidly in dry ice and stored at −80 °C until the analysis of mRNA gene expression. The other gonad lobule was collected, immersed in 10% formaldehyde fixative solution (Leica Biosystems, Spain) and stored until histological analyses.

2.3. Sex steroid ELISA analysis

With commercial ELISA kits (Tecan, Hamburg, Germany) that had previously been validated for tilapia plasma samples, the E_2 and T concentrations were determined in all of the adult fish plasma samples (270 dpf) (de Alba et al., 2019).

2.4. Histological analyses

Gonad samples were fixed for 24 h in 10% formaldehyde solution before transferring to 70% ethanol and after dehydration, embedding in paraplast (Leyca Bioystems, Spain). Hematoxylin and eosin (HE) were used to stain sections of around 5 μm. A light microscope with a camera (Axiolab, Zeiss, Oberkochen, Germany) was used to examine the slides (Photometrics Coolsnap, Roper Scientific, Buckinghamshire, UK). Female and male sex was determined by the identification of oocytes or spermatocytes, respectively (Fig. 2).

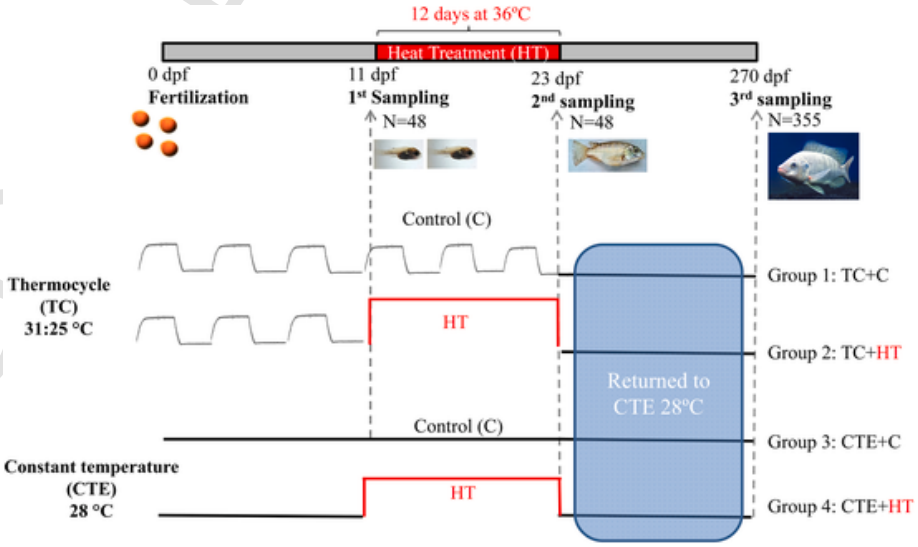

Fig. 1. Schematic representation of the experimental design. Fertilized eggs (stage 1) were divided into two groups reared in different thermal regimes: thermocycle (TC) of 31 °C:25 °C day:night *vs.* a constant temperature of 28 °C (CTE). Embryos and larvae were left nder these conditions from 0 to 11 days post fertilization (dpf). At the end of this period, the larvae from each temperature regime were either subjected to heat treatment (HT, 36 °C for 12 days) or left at the same rearing temperatures from 11 to 23 dpf (Control, C). Then after this period, all the groups remained at constant temperature (28 °C) until 270 dpf.

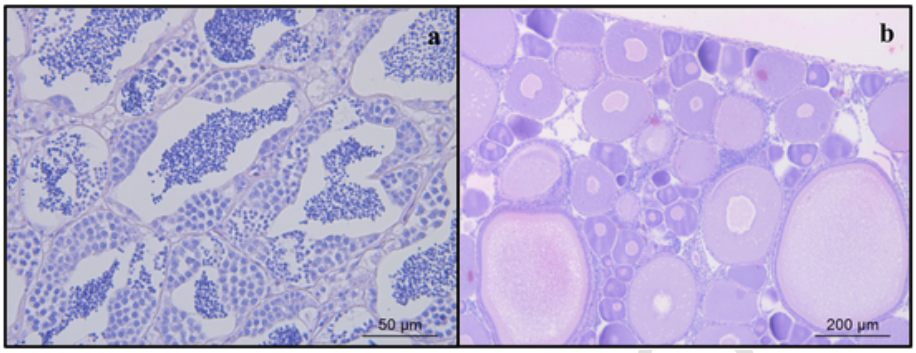

Fig. 2. Representative histological section of the testis (A) and ovary (B) of Nile tilapia juveniles in the reproductive stage. Longitudinal cut. Staining: H-E. Scale bar: (A) x 50 and (B) x 200.

2.5. Real-time RT-PCR analysis

With the use of a tissue homogenizer (TissueLyser LT, Qiagen, Hilden, Germany), t larvae and gonad samples were homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, USA). After dissolving the RNA in sterile DEPC water (Invitrogen, CA, USA), the RNA concentration and purity were determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific). Prior to retrotranscription, genomic DNA contamination was removed from RNA (1 g) by treating it with 1 U of DNase I (Thermo Fisher). A thermocycler (MiniAmp Plus, Applied Biosystems, Foster City, USA) and commercial reverse transcriptase kit (QSCRIPT cDNA Synthesis Kit, Quantabio, Beverly, USA) were used to synthesize cDNA. Before the expression analysis, all of the cDNa samples were diluted (1:10) in nuclease-free water (Thermo Fisher Scientific). Using Perfecta® SYBR® Green Fastmix (Quantabio) in a final volume of 20 µl for each qPCR reaction, quantitative PCR (qPCR) reactions were performed. With the use of a light thermocycler (7500 RT-PCR system, Applied Biosystems, Foster City, USA), all the samples were run in duplicate with the following steps: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The thermocycler ran melting curves right away after the amplification phase to ensure that the reaction was selective and that just one DNA species was amplified. Primer 3 Plus software was used to create primers (Table 1). (Untergasser et al., 2012). Dilution curves were used to determine the primer concentration and confirm that all of the primers had relative amplification efficiencies between 90% and 110%. (Table 1). A 500 nM final concentration of each primer was used. Because *Beta-Actin (βactin)* was consistent and unaffected by temperature (coefficient of variation 5%), it was used as a reference gene (Vanhauwaert et al., 2014). Then, using the 2–ΔΔCt technique, the relative expression of all the genes was determined (Livak and Schmittgen, 2001).

Table 1
Primer sequences used for the quantitative PCR analyses.

Gene	F/R	Sequence (5'-3')	Ensembl/GenBank Accession number
amh	F	ACGACGCGCAAAGAAAACTG	ENSONIG00000004781
	R	TAATGTTTGCGCTGCTTGGG	
cyp19a1a	F	TTGCACAAAACCACGGTGAG	NM_001279586.1
	R	ACGTGCGGGTTTTGTTTGAG	
ara	F	CAGCCCTATGTCCTTGCTTACCAG	XM_005467840.4
	R	CGCTGGTCATTGAAAATCAGGTCT	
era	F	TATGTGCCCAGCGACAAATC	NM_001279770.1
	R	CGTTTTTCACGCCGCAAAAC	
sox9a	F	GTGTTGAAGGGTTACGACTGGACG	XM_003450119.4
	R	CCGTTCTTGACAGACTTTCTCCGC	
foxl2	F	AGCATTTCACCGATCGAGAC	NM_001279778.1
	R	CATTGCGCACACACCAAAAC	
dmrt1a	F	CGGATTGCAGCGGACCGA	XM_013270912.3
	R	GGACAGAGACACAGGACTAC	
βactin	F	TGGTGGGTATGGGTCAGAAAG	ENSONIG00000008505
	R	CTGTTGGCTTTGGGGTTCA	

2.6. Data analysis

The results are expressed as mean ± SEM. For all the tests, the significance threshold was set at $\alpha = 0.05$. To perform the statistical analyses, the SPSS software (v. 19.0, IBM, Armonk, NY, USA) was used. The Kolmogrov-Sminorv test and Levene's test were used to determine and confirm the homoscedasticity and normality of the data, respectively. For the 11 dpf data, separate Student's t-test (7 in total) were run for expression of each gene analyzed (*amh*, *ara*, *sox9a*, *dmrt1a*, *cyp19a1a*, *era* and *foxl2*). For the 24 dpf and juveniles data, separate two-way ANOVAs (17 in total) were run for each factor analyzed (24 dpf data: survival rate and *amh*, *ara*, *sox9a*, *dmrt1a*, *cyp19a1a*, *era* and *foxl2* expression; juveniles data: sex ratio, weight, plasma concentrations of E_2 and T, and gonadal *amh* and *cyp19a1a* expression). In the two-way ANOVA, the significant effects of rearing temperature regime (R), HT and their interaction were tested. Following the two-way ANOVA, Duncan's *post hoc* analysis was used to determine whether there were any significant differences between the four experimental groups (TC + C, TC + HT, CTE + C and CTE + HT).

3. Results

3.1. Effects of the rearing temperature regime on the survival rate and gene expression on 11 dpf

The Nile tilapia larvae survival rate from 0 to 11 dpf did not show any statistically significant differences between rearing temperature regimes (*t*-test, $P = 0.517$, Table A1), with values of 51.6 ± 3.9 and 45.3 ± 4.4% for the TC and CTE groups, respectively. In the gene expression analysis, significant differences were detected in the mRNA expression of some of the genes involved in gonadal development (Fig. 3) (*t*-test, $P < 0.05$; Table A1). The larvae reared in CTE showed a higher mRNA expression of *ara* than those reared in the TC (Fig. 3c) (*t*-test, $P = 0.028$; Table A1). On the contrary, the expressions of *cyp19a1a*, *era* and *foxl2* were higher in the larvae reared in the TC than in CTE (Fig. 3b, d, f) (*t*-test, $P < 0.05$; Table A1). Finally, no statistically significant differences between both groups were detected for *amh*, *sox9a* and *dmrt1a* (Fig. 3a, e, g) ($P > 0.05$; Table A1).

3.2. Effects of the rearing temperature regime and heat treatment on the survival rate and gene expression on 24 dpf

The Nile tilapia larvae's survival rate after HT showed statistically significant differences between the experimental groups (Fig. 4) (two-way ANOVA, $P < 0.004$; Table A1). Survival was influenced by both the HT (two-way ANOVA, $P = 0.001$) and by the rearing temperature

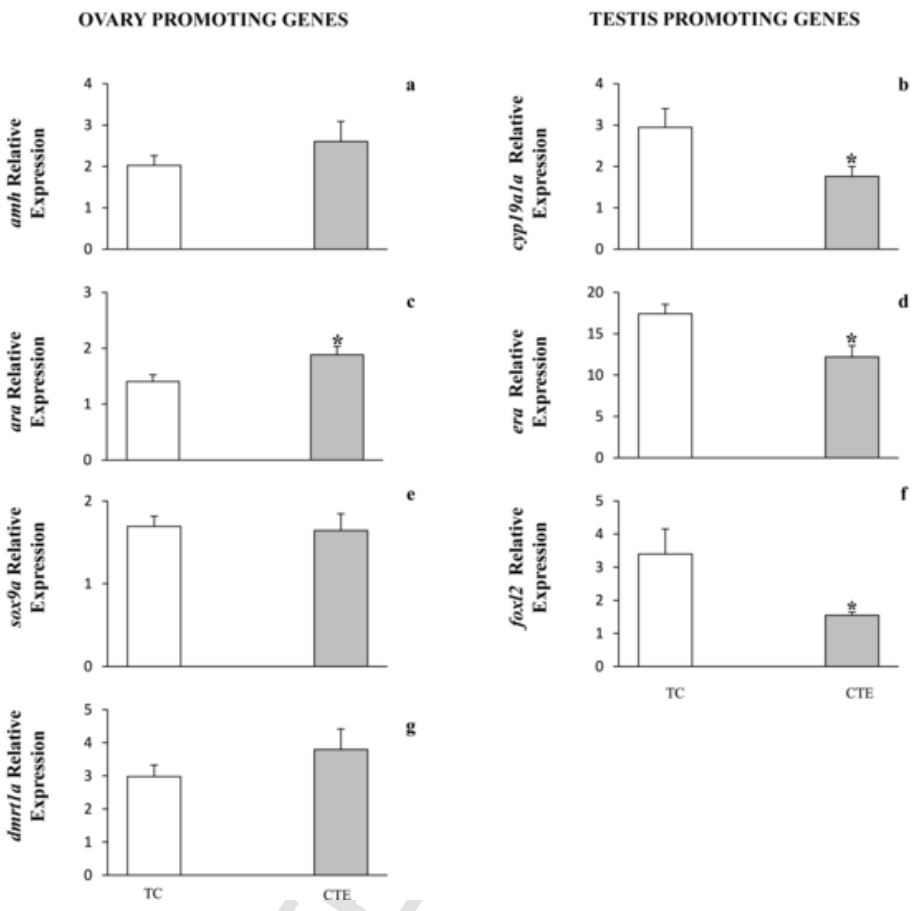

Fig. 3. Relative mRNA expression of *amh* (A), *cyp19a1a* (B), *ara* (C), *era* (D), *sox9a* (E), *foxl2* (F) and *dmrt1a* (G) in Nile tilapia larvae on 11 days post fertilization (dpf) and reared in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25 ± 0.2 °C) (white bars) *vs.* constant temperature (CTE, 28 ± 0.2 °C) (gray bars). Asterisks denote significant differences between rearing temperature regimes (Student's t-test, $P < 0.05$). Data (n = 12) are represented as mean ± SEM.

regime (two-way ANOVA, $P = 0.047$) (Fig. 4 and Table A1). The lowest survival rate was observed in the larvae from the CTE + HT group (49.4 ± 3.5%), while the highest survival rate appeared in the C groups under both temperature conditions (91.8 ± 1.8% and 81.1 ± 4.3% for TC and CTE, respectively) and in the TC group exposed to HT (72.2 ± 5.9%).

On 24 dpf, all the genes showed significant intergroup differences (Fig. 5) (two-way ANOVA, $P < 0.05$; Table A1). The expressions of *ara, sox9a*, and *dmrt1a* were higher in the CTE + HT group (Fig. 5c, e, g) (two-way ANOVA, $P < 0.05$; Table A1). While the expressions of *ara* and *dmrt1a* were influenced by HT (higher in HT *vs.* C), *sox9a* expression was influenced by temperature regime (higher in the animals reared in CTE *vs.* TC, regardless of them being subjected or not to HT) (Fig. 5c, e, g) (two-way ANOVA, $P < 0.05$; Table A1). A significant interaction between temperature regime and HT was found in the expressions of genes *ara* and *sox9a* (two-way ANOVA, $P < 0.05$; Table 1). The *amh* expression was significantly higher in the CTE + C group (Fig. 5a) (two-way ANOVA, $P = 0.042$; Table A1), while the *cyp19a1* expression was higher in the TC + C group (Fig. 5b) (two-way ANOVA, $P = 0.004$; Table A1) and was influenced by HT (higher in the Control groups *vs.* HT) (two-way ANOVA, $P = 0.009$; Table A1). Finally, the expressions of *era* and *foxl2* presented similar patterns to one another, with a higher expression in TC + C than in the CTE + HT group (Fig. 5d, f) (two-way ANOVA, $P < 0.05$; Table A1). The statistical analyses also revealed a significant effect of temperature regime because the larvae reared at TC in early development stages had higher *era* and *foxl2* expressions than those reared in CTE (two-way ANOVA, $P < 0.05$; Table A1).

3.3. Effects of the rearing temperature regime and heat treatment on sex ratio, weight, plasma sex steroids and gonadal gene expression in the juvenile stage

Tilapia embryo and larvae were reared under the four experimental conditions until the juvenile stage (270 dpf) when the sex ratio, whole body weight, plasma levels of sex steroids and mRNA expression of the genes involved in the synthesis of sex steroids in gonads were measured (Figs. 6 and 7).

For the sex ratio, the statistical analysis showed differences between experimental groups (Fig. 6) (two-way ANOVA, $P = 0.003$; Table A1). The fish reared in TC + C had a higher proportion of females (56.2 ± 4.1%) than both the groups reared in CTE (38.1 ± 7.7 and 22.2 ± 2.7% for CTE + C and CTE + HT, respectively). However, no differences in the sex ratio were found between both groups from the TC: TC + C and TC + HT (42.1 ± 3.3%). The statistical analysis also showed that rearing tilapia larvae in the TC on the first days of development led to a higher proportion of females than in CTE (two-way ANOVA, $P = 0.002$; Table A1). A significant effect of HT was also noted with a higher proportion of males in the groups exposed to HT than those maintained in rearing temperature regimes (C) (two-way ANOVA, $P = 0.01$; Table A1).

About tilapia juveniles' weight, the statistical analysis detected significant differences between experimental groups with a similar pattern in both sexes (Fig. 7a–b) (two-way ANOVA, $P < 0.05$, Table A1). The juveniles from CTE + C and CTE + HT were heavier than those from TC + C, and for both males (Fig. 7a) and females (Fig. 7b). No significant differences were detected between fish weight from both TC

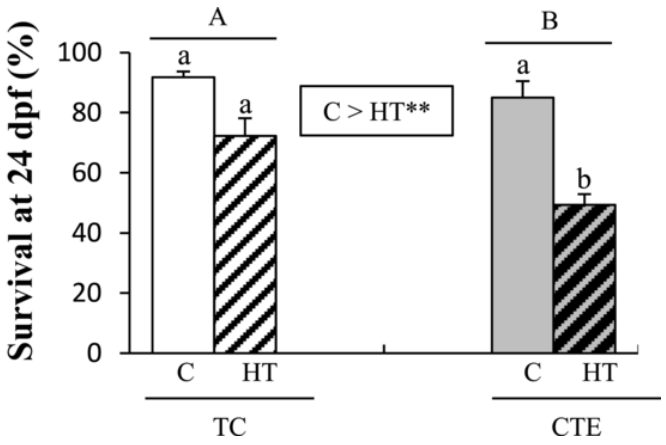

Fig. 4. Effect of heat treatment on the Nile tilapia larvae survival rate. Larvae were reared from 0 to 11 days post fertilization (dpf) in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25 ± 0.2 ˚C) (white bars) *vs.* constant temperature (CTE, 28 ± 0.2 ˚C) (gray bars). From 11 to 23 dpf, the larvae from each group were either exposed to a heat treatment of 36 ˚C (HT, stripped bars) or maintained in the same rearing regime as before (control group, C; solid bars). Survival was evaluated on 23 dpf. Different lower case letters indicate significant differences between experimental groups. Different upper case letters and asterisks denote significant differences between rearing temperature regimes and heat treatment effects, respectively (two-way ANOVA, $P < 0.05$). Data (n = 4) are represented as mean ± SEM.

groups (two-way ANOVA, $P > 0.05$, Table A1). In both sexes, weight was influenced by the temperature regime with higher values in the fish reared under CTE than in the TC (two-way ANOVA, $P < 0.001$, Table A1). The statistical analyses also revealed a significant effect of HT ($P = 0.005$) on females' weight, with higher values for the fish exposed to HT than the C groups (Fig. 7b) (two-way ANOVA, $P < 0.05$, Table A1).

Plasma levels of sex steroids were measured in all the individuals (Fig. 7c–f). Regarding E_2, the analysis detected statistical differences between the experimental groups in females, but not in males (Fig. 7c–d) (two-way ANOVA, $P < 0.05$; Table A1). The females from TC + C showed higher plasma levels of E_2 (28.5 ± 1.2 ng/ml) than the fish reared in CTE + HT (21.7 ± 1.8 ng/ml) (Fig. 7d) (two-way ANOVA, $P = 0.019$, Table 1). As regards T, the plasma levels displayed similar statistical differences between groups for both sexes (Fig. 7e–f) (two-way ANOVA, $P < 0.05$; Table 1). The tilapia from the CTE + HT group obtained the highest T values (9.0 ± 0.5 and 9.15 ± 0.9 ng/ml for males and females, respectively), while the fish reared in TC + C presented the lowest levels (5.2 ± 0.5 and 5.5 ± 0.5 ng/ml for males and females, respectively) (two-way ANOVA, $P < 0.05$; Table A1). However, no significant differences in T were detected between both groups for each temperature regime (TC or CTE) in males or females (Fig. 7c–f). Regardless of the rearing temperature regime, an effect of HT was observed on females' E_2 production, with HT lowering its plasma levels (two-way ANOVA, $P = 0.02$; Table 1). Moreover, an effect of the rearing temperature regime was observed in T production because the fish of both sexes reared in CTE had higher levels than those reared in the TC (two-way ANOVA, $P < 0.001$; Table A1).

Finally, the analysis also revealed statistically significant differences in the relative mRNA expressions of *amh* and *cyp19a1a* in tilapia juveniles' testes and ovaries, respectively (Fig. 7g–h) (two-way ANOVA, $P < 0.05$; Table A1). The highest *amh* expression was detected in the males reared in CTE, with the lowest one in the males reared in the TC and subjected to HT (Fig. 7g) (two-way ANOVA, $P = 0.039$; Table A1). The highest *cyp19a1a* expression for females was for the TC + C group, with the lowest in the females reared in CTE (Fig. 7h) (two-way ANOVA, $P < 0.05$; Table A1). The two-way ANOVA revealed that the males reared in CTE had a higher *amh* expression than those in the TC regardless of HT (Fig. 7g). The opposite effect on *cyp19a1a* expression occurred in females, with higher levels in the fish reared in the TC *vs.* CTE (Fig. 7h) (two-way ANOVA, $P < 0.05$; Table A1).

4. Discussion

The present research work reveals the strong effect of daily temperature cycles on Nile tilapia sex determination and differentiation, and also their influence on the female-male sex reversal process by high temperature treatment. TCs increased the survival rate against HT and also up-regulated the expression of the genes related to female sexual differentiation on both 11 and 24 dpf. HT modified the expression patterns of the sex differentiation genes only in the larvae reared in CTE by increasing the expression of the genes involved in testicular differentiation and decreasing the expression of the genes related to ovarian differentiation. This modification was not observed in the larvae reared in the TC, which showed less susceptibility to HT. The differences observed during larval sex differentiation between both rearing temperature regimes were maintained in the juvenile fish. In this stage, daily TCs increased the female proportion, which presented higher ovarian *cyp19a1a* expression and plasma E_2 levels. In addition, HT induced a more marked presence of males with higher plasma T and testicular *amh* expression levels only in the CTE group, but not in the TC group, which presented lower sensitivity to high temperature sex reversal.

In Nile tilapia aquaculture, high temperature treatments in the larval stage are one of the most common methods for obtaining a higher proportion of males (Baroiller et al., 2009; de Alba et al., 2021b). However, this process entails some disadvantages, such as increased mortality (de Alba et al., 2021b, 2021c). In the present study, a higher larval survival rate after HT was observed when larvae were reared in the TC compared to those reared in CTE. To date, the effect of TCs on thermal tolerance and survival after thermal shock has been studied in other fish species with different results. In Atlantic salmon (*Salmo salar*), very mild effects of TCs were observed on their thermal tolerance response (Corey et al., 2017; Gallant et al., 2017). However in zebrafish, diurnal TCs increased fish thermal tolerance compared to those reared at constant temperatures (Cortemeglia and Beitinger, 2005; Schaefer and Ryan, 2006; Xia et al., 2016; Wang and Xia, 2019; de Alba et al., 2022), as herein observed in tilapia. One hypothesis to explain the different acute thermal tolerance of fish species would be that it might be influenced by the temperature amplitude and rate of temperature change of the diel thermal cycle (Schaefer and Ryan, 2006; Xia et al., 2016; Gallant et al., 2017). Such heat hardening seems to be related to acclimation to cyclical thermal environments, especially when fish are subjected to temperatures peaks higher than the temperatures faced by fish reared in stable or constant thermal environments, and even when the average temperature is the same in all groups as happened in the present research (28.1 ± 0.1 ˚C in TC) (Schaefer and Ryan, 2006).

The two rearing temperature regimes herein applied not only impacted survival during larval development, but also influenced the expression of several genes. The analyzed genes have previously been identified as molecular markers involved in ovarian and testicular differentiation in Nile tilapia (Lu et al., 2022). In our study, on 11 and 24 dpf we observed that the tilapia larvae reared in TCs presented higher values in the expression of the genes related to ovarian differentiation, such as *cyp19a1a*, *foxl2* and *era*. In contrast, constant temperature promoted the expression of testicular differentiation factors like *ara*, *sox9a* and *dmrt1*. These results agree with those obtained by Villamizar et al. (2012), who showed that zebrafish larvae reared at constant temperature presented lower *cyp19a* expression and higher *amh* expression levels than the larvae reared in TCs. The effects of ecologically fluctuating temperatures on the expression of sexual differentiation mechanisms has been extensively investigated in reptiles showing that different sex genes respond in a specific way to the temperature regime (Breitenbach et al., 2022). In other research works conducted in Nile tilapia, the ef-

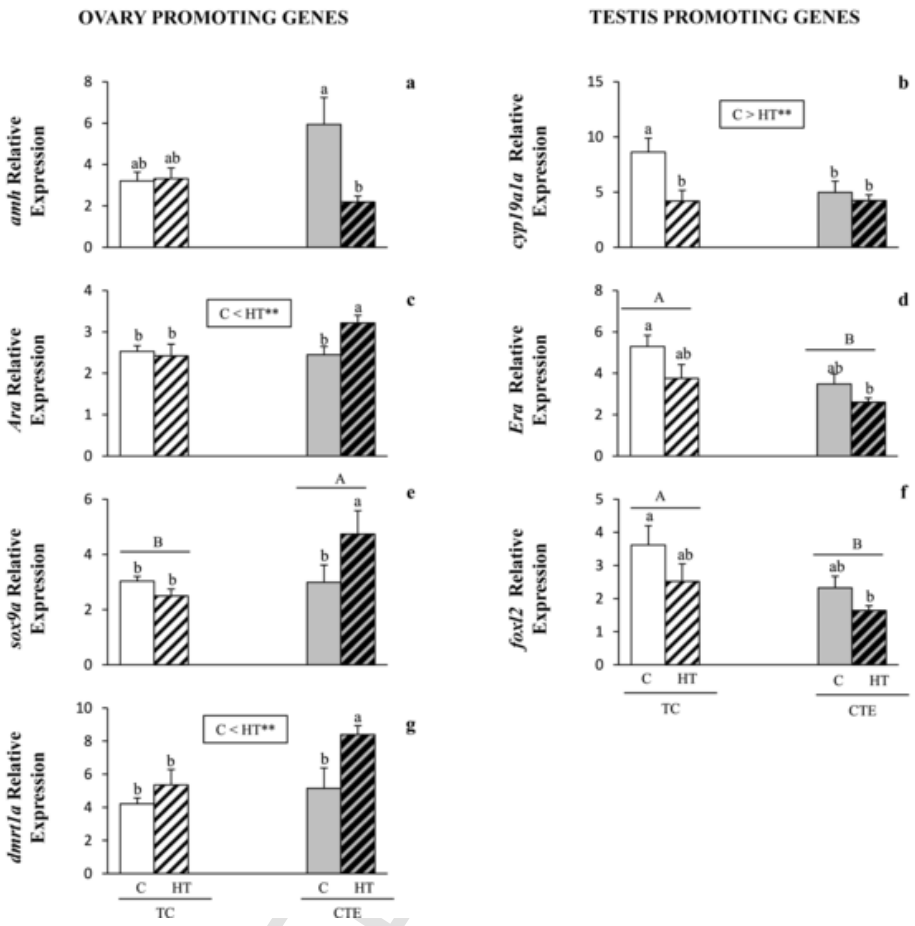

Fig. 5. Effect of heat treatment on the relative mRNA expression of *amh* (A), *cyp19a1a* (B), *ara* (C), *era* (D), *sox9a* (E), *foxl2* (F) and *dmrt1a* (G) on 24 days post fertilization (dpf) in Nile tilapia larvae. Larvae were reared from 0 to 11 dpf in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25 ± 0.2 ˚C) (white bars) *vs.* constant temperature (CTE, 28 ± 0.2 ˚C) (gray bars). From 11 to 23 dpf, the larvae from each group were either exposed to a heat treatment of 36 ˚C (HT, stripped bars) or maintained in the same rearing regime as before (control group, C; solid bars). Different lower case letters indicate significant differences between experimental groups. Different upper case letters and asterisks denote significant differences between rearing temperature regimes and heat treatment effects, respectively (two-way ANOVA, $P < 0.05$). Data (n = 12) are represented as mean ± SEM.

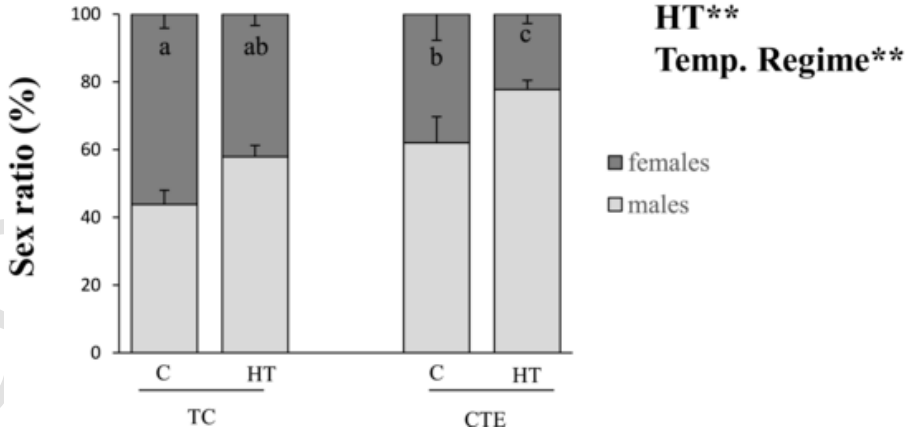

Fig. 6. Effect of heat treatment on the sex ratio (%) of the Nile tilapia juveniles (270 days post fertilization, dpf) reared from 0 to 11 dpf in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25 ± 0.2 ˚C) *vs.* constant temperature (CTE, 28 ± 0.2 ˚C). From 11 to 23 dpf, the larvae from each group were either exposed to a heat treatment of 36 ˚C (HT) or maintained in the same rearing regime as before (control group, C). Different lower case letters indicate significant differences between experimental groups of males (light bars) and females (dark bars), respectively. Different asterisks denote significant differences between rearing temperature regimes and heat treatment effects, respectively (two-way ANOVA, $P < 0.05$). Data (n = 92 for TC + C, 95 for TC + HT, 88 for CTE + C and 80 for CTE + HT; N = 335) are represented as mean ± SEM.

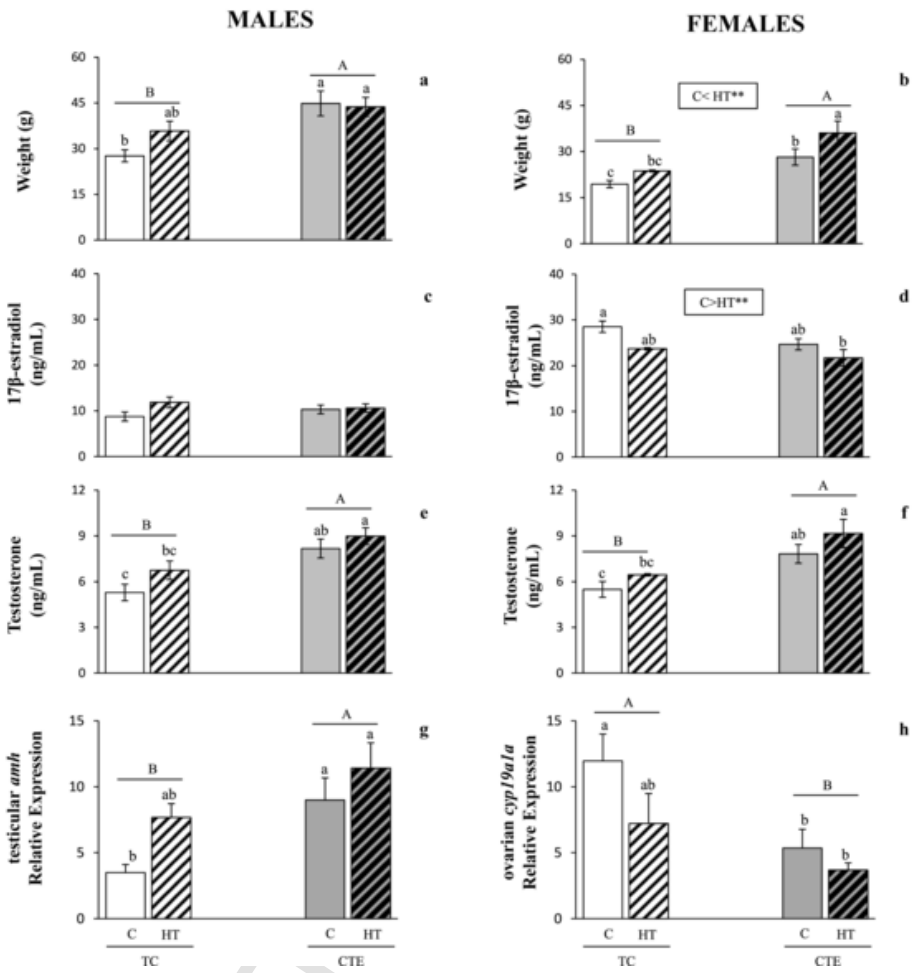

Fig. 7. Effect of heat treatment on weight (A–B), plasma 17β-estradiol (C–D), plasma testosterone (E–F), testicular *amh* (G) and *cyp19a1a* (H) relative mRNA expression in male (left panels) and female (right panels) Nile tilapia juveniles (270 days post fertilization, dpf). Larvae were reared from 0 to 11 dpf in two temperature regimes: thermocycle (TC, thermophase:cryophase 31:25 ± 0.2 °C) (white bars) *vs.* constant temperature (CTE, 28 ± 0.2 °C) (gray bars). From 11 to 23 dpf, the larvae from each group were either exposed to a heat treatment of 36 °C (HT, stripped bars) or maintained in the same rearing regime as before (control group, C; solid bars). Different lower case letters indicate significant differences between experimental groups. Different upper case letters and asterisks denote significant differences between rearing temperature regimes and heat treatment effects, respectively (two-way ANOVA, $P < 0.05$). Data (n = 12) are represented as mean ± SEM.

fect of HT on these molecular markers has been observed from the first exposure days and in very early development stages (from 10 dpf) (Li et al., 2014; Lu et al., 2022). In tilapia, the *dmrt1* and *amh* expressions are up-regulated by HT that, in turn, brings about an increase in the expression of the other genes involved in testicular development, such as *sox9* and *11β-hydroxylase* (a cytochrome P450 enzyme involved in steroid hormone synthesis) (Iriji et al., 2008). The result of the activation of testicular pathway genes is the inhibition of ovarian differentiating genes, such as *cyp19a* and *foxl2* (Lu et al., 2022). This agrees with our results because the larvae reared in CTE and exposed to high temperature had higher *sox9a, ara* and *dmrt1a* expressions. In addition, the up-regulation of the above genes was followed by a consequent suppression of gene expression for the factors involved in the ovarian pathway (*foxl2* and *era*), but only for the CTE-reared larvae. These changes in the up-regulation of male sex differentiation genes and the suppression of female sex differentiation genes were not observed in the TC group, except for *cyp19a1a* expression, which was inhibited by HT in the TC and CTE groups. Therefore in general, the larvae reared in the TC were less susceptible to the effects of HT on the expression of the genes involved in sexual differentiation pathways. This may be related to the effects of TCs on survival, which point out greater thermotolerance for the fish reared under these conditions. Despite some studies supporting this hypothesis (Li et al., 2014), further research is needed.

Therefore, the combined effect of rearing temperature regime and HT led to differences in the expression of sex differentiation genes. These differences ultimately impacted the sex ratio of the tilapia juveniles from the four tested groups. Although Nile tilapia are exposed to the daily TCs caused by alternations between day and night in their natural environment (Lopes and Henry-Silva, 2014), the effect of TCs on gonadal differentiation and sex ratio have not yet been studied in depth. In previous studies, similar feminizing effects of TCs on sex ratio have been observed in other fish species, such as Senegalese sole (Blanco Vives et al., 2011) and zebrafish (Villamizar et al., 2012). With tilapia, the first study of the influence of daily TCs on phenotypic differentiation was performed in blue tilapia (*Oreochromis aureus*) by Baras et al. (2000). In this previous research work, TCs led to a slightly lower proportion of males compared to constant temperatures. In our study, TCs more considerably increased the proportion of females. This clearer feminizing response of TCs herein observed may be due to differences in the environmental response of the sex determination system among tilapia species (Prugirin et al., 1975). This could also be explained by the age of the larvae exposed to TCs. According to the research by Baras et al. (2000), larvae were exposed from 10 dpf. In our study, they were exposed from the first hours of fertilization when still in embryo stages. Thus our results could indicate that early development stages are also sensitive to temperature changes by producing irreversible effects on

sexual determination and differentiation mechanisms. This hypothesis agrees with previous research performed in Nile tilapia, which describes an earlier thermal sensitivity window during embryogenesis from 12 hpf to hatching (Rougeot et al., 2008). In the present study we also observed an effect of rearing temperatures (TC *vs*. CTE) on the response to HT. In Nile tilapia, as in other fish species, exposure to high temperatures during the sexual differentiation period can induce profound effects on individuals' phenotype by shifting from females to functional phenotypic males (Baroiller et al., 2009). Although the effect of TCs in early developmental stages has been observed in the sexual differentiation of some fish, their influence on the female-male sex reversal process by high temperature has gone unnoticed. The present study shows, for the first time, that tilapia reared on the first days of life in daily TCs are less sensitive to the masculinizing effect of HT than those reared at constant temperature. Previous studies have suggested that the degree of sensitivity to sex reversal by high temperature (thermosensitivity) is considerably influenced by the rearing temperature regime during development (Baras et al., 2000; Baroiller and D'Cotta 2001; Tessema et al., 2006). For example, different Nile tilapia populations adapted to high or low temperature environments have shown greater or lesser variability, respectively, in their response to HT in sex ratio terms (Bezault et al., 2007). According to our study results, further research is needed to investigate in depth the molecular mechanisms involved in the changes in thermosensitivity caused by implementing TCs during development.

Finally in the present research, the rearing temperature regimes implemented during development also impacted juveniles' sex steroid production. The females reared in TCs showed higher *cyp19a1a* expression and E_2 plasma levels, while the females reared in CTE had higher T levels. The males reared in CTE presented higher *amh* expression and plasma T levels than those reared in TCs. These results are similar to those found for Senegalese sole, in which the fish reared in TCs presented higher E_2 and *cyp19a1a* expression levels than those left at constant temperatures that, in turn, showed higher T levels (Blanco-Vives et al., 2011). However, that study was unable to differentiate according to individuals' gender. Our study observed that the T levels in the males and females from the different experimental groups presented a similar pattern, which coincides with the research carried out in other fish species (Borg, 1994; D'Cotta et al., 2001; Lokman et al., 2002). Our results also showed that the effect of HT on sex determination mechanisms in early developmental stages remained in the juvenile stage. From the first hours of fertilization, Nile tilapia presents sensitivity to temperature, and exposure to high temperatures can lead to the inhibition of the cyp19a1a enzyme (Baroiller et al., 1995; Li et al., 2014). This inhibition blocks the conversion of T into E_2 by lowering E_2 levels and, thus, inducing testicular differentiation (Baroiller et al., 2009; Mahmoud et al., 2020). The results suggest that TCs can reduce the suppressive effects of aromatase activity caused by HT during the sex differentiation period by limiting the female-to-male sex reversal process. Studies carried out on Nile tilapia have shown that the plasma level of sex steroids considerably impacts the animal's physiology and plays a role in the sexual dimorphism of growth by regulating the expression of growth factors (Yue et al., 2016). This could explain the results herein obtained because the females and males reared at constant temperature and exposed to HT had higher T levels and, thus, a heavier body weight than those reared in TCs and not exposed to HT.

5. Conclusions

To conclude, the present research demonstrates that implementing TCs from early development stages can promote ovarian differentiation. TCs increase the expression of the genes involved in ovarian differentiation and, consequently, plasma E_2 levels resulting proportion of females. In addition, TCs can reduce sensitivity to the masculinizing effect of HT by reducing the up-regulation of the genes involved in testicular differentiation and, thus, the plasma T levels and the resulting proportion of males. These results suggest that the mechanisms involved in sensitivity to the female-to-male sex reversal process may be related to certain factors associated with individuals' heat tolerance. These findings should be considered when discussing the influence of environmental temperature on Nile tilapia sex determination and sexual differentiation.

Data accessibility

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Credit author statement

GA, JFLO and FJSV conceived and designed the experiments, and wrote the manuscript; GA and MC performed the experiments; GA and JFLO analyzed the data; FJSV, JFLO and MAE provided funding.

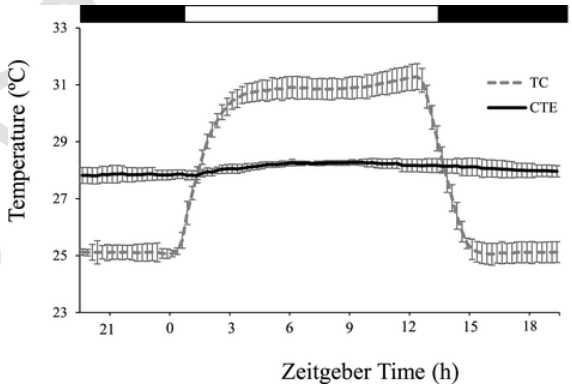

Fig. A1. Daily average water temperature (mean ± S.D.) throughout the experiments in the two temperature regimes herein tested: a thermocycle (TC) of 31 °C:25 °C (dashed line) or a constant temperature (CTE) of 28 °C (continuous line). The time scale is expressed as Zeitgeber Time (ZT), where ZT0 h corresponds to light onset.

Table A1
Statistic values obtained in the Student's t-test and two-way ANOVAs performed for all the variables measured in the experiments. The p values (*P*) and F-Statistic (F) are reported. Gray highlights the statistically significant results ($P < 0.05$).

Variable			T-test	T-test
			F	P
11 DPF	**Survival rate (%)**		0.517	0.499
	Relative expression	*amh*	0.636	0.438
		cyp19a1a	5.322	0.032
		Ara	5.685	0.028
		Era	8.489	0.008
		sox9a	0.037	0.849
		foxl2	5.264	0.033
		dmrt1a	1.208	0.285

Variable			Two-way ANOVA					
			Corrected model		Temperature regime		Heat treatment	
			F	P	F	P	F	P
24 DPF	Survival rate (%)		7.821	0.004	4.909	0.047	17.099	0.0
	Relative expression	*amh*	3.029	0.042	0.769	0.387	3.993	0.0
		cyp19a1a	5.316	0.004	2.776	0.105	7.597	0.0
		Ara	3.234	0.034	0.760	0.389	4.212	0.0
		Era	3.433	0.028	5.108	0.031	3.419	0.0
		foxl2	3.278	0.032	5.349	0.027	3.610	0.0
		sox9a	3.912	0.017	5.229	0.029	1.607	0.2
		dmrt1a	2.952	0.047	3.618	0.066	4.339	0.0
JUVENILES	Sex ratio (%)		8.209	0.003	15.228	0.002	9.370	0.0
	Weight	Male	5.047	0.002	13.668	0.000	1.080	0.3
		Female	10.118	0.000	24.647	0.000	8.143	0.0
	Sex steroids T	Male	5.704	0.000	14.895	0.000	2.920	0.0
		Female	5.376	0.002	13.832	0.001	1.704	0.1
	E_2	Male	1.053	0.371	0.017	0.898	2.240	0.1
		Female	3.458	0.019	3.120	0.080	5.520	0.0
	Relative expression	*amh*	3.149	0.039	6.659	0.015	3.414	0.0
		cyp19a1a	3.860	0.017	7.487	0.009	3.008	0.0

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Uncited references

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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