**ORIGINAL ARTICLE**

# Thyroid axis participates in high-temperature-induced male sex reversal through its activation by the stress response

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## Abstract
Environmental changes alter the sex fate in about 15% of vertebrate orders, mainly in ectotherms such as fish and reptiles. However, the effects of temperature changes on the endocrine and molecular processes controlling gonadal sex determination are not fully understood. Here, we provide evidence that thyroid hormones (THs) act as co-players in heat-induced masculinization through interactions with the stress axis to promote testicular development. We first demonstrated that the thyroid axis (through thyroid-related genes and T3 levels) is highly active in males during the gonadal development in medaka (*Oryzias latipes*). Similarly, T3 treatments promoted female-to-male sex reversal in XX embryos. Subsequently, embryonic exposure to temperature-induced stress up-regulated the genes related to the thyroid and stress axes with a final increase in T3 levels. In this context, we show that blocking the stress axis response by the loss of function of the corticotropin-releasing hormone receptors suppresses thyroid-stimulating hormone expression, therefore, heat-induced activation of the thyroid axis. Thus, our data showed that early activation of the stress axis and, in consequence, the TH axis, too, leaves us with that both being important endocrine players in inducing female-to-male reversal, which can help predict possible upcoming physiological impacts of global warming on fish populations.

## Abbreviations
| | |
|---|---|
| amh | Anti-müllerian hormone |
| amhrII | Anti-müllerian hormone receptor type II |
| ar | Androgen receptor |
| BPI | Brain-pituitary-interrenal |
| BPT | Brain-pituitary-thyroid |
| CRH | Corticotropin-releasing hormone |
| crhb | Corticotropin-releasing hormone b |
| Crhr2 | Corticotropin-releasing hormone receptor 2 |
| cyp19a1a | Cytochrome P450, family 19, subfamily A, polypeptide 1a; ovary marker |
| dio1 | Deiodinase type 1 |

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| dio2 | Deiodinase type 2 |
| dio3 | Deiodinase type 3 |
| foxl2 | Forkhead box protein L2 |
| gsdf | Gonadal soma derived factor; testicular marker |
| MM | Methimazole |
| R | RU486; mifepristone |
| T | Testosterone |
| T2 | Diiodothyronine; 3,5- diiodotyrosine |
| T3 | Triiodothyronine; 3, 5, 3′-L-triiodothyronine |
| T4 | Thyroxine; 3, 5, 3′, 5′-L-tetra-iodothyronine |
| THs | Thyroid hormones |
| TRH | Thyrotropin-releasing hormone |
| TSH | Thyroid-stimulating hormone |
| thra | Thyroid hormone receptors a |
| thrb | Thyroid hormone receptors b |
| TRs | Thyroid receptors |
| tshba | Thyroid-stimulating hormone subunit beta a |
| tshr | Thyroid-stimulating hormone receptors |

## Introduction

An enormous number of sex-determining mechanisms can control the fate of biological sex. These mechanisms range from genomic differences to environmental changes or a combination of both [1]. Fish are the vertebrate taxon with the greatest diversity of sex determination strategies [2]. In this group, changes in the environment, such as temperature, during embryonic or larval stages can trigger testis differentiation [3]. In light of climate change [4], it is critical to understand the mechanisms that trigger the fate of sex determination through increases in temperature changes, and this could negatively impact fish reproduction in the future.

Changes in water temperature is one of the most studied physical stressors that can change sex bias in fish. Higher temperatures have been shown to activation of the brain-pituitary-interrenal axis [5], increasing plasma cortisol levels [6–9]. Moreover, the exogenous application of cortisol promotes the development of testis and therefore sex reversal/change [6–8, 10, 11]. High level of cortisol promotes androgen synthesis [12, 13], masculinizing the bipotential gonad as a by-product effect of stress [14]. Although the involvement of stress in heat-induced masculinization has been characterized in recent years, the endocrine and molecular processes controlling temperature-induced sex change are not fully understood.

Thyroid hormones (THs) have been proposed as stress modifiers due to their ability to modulate metabolic and osmotic processes in fish [15, 16]. The involvement of THs in response to different types of stress (e.g., changes in water salinity, chemical exposure, handling, cold, and heat stress) and adaptations to stress have been reported [15–17].

Furthermore, during early gonadal development, exposure of zebrafish to perchlorate, an inhibitor of THs synthesis, induced a female bias [18], and conversely, T4 biased the sex ratio toward males [19]. However, the role of THs in stress-induced sex reversal remains unexplored.

Interaction between the thyroid and stress axes has been studied in mammals, birds and amphibians. In rats subjected to physical stress, it was observed that after 15 days THs levels decreased [20]. Moreover, it was also observed in mice that thyrotropes (αTSH cells) express corticotropin-releasing hormone (CRH) receptors [21]. Interestingly, in chickens, CRH induced the release of thyroid-stimulating hormone subunit beta a (*tshba*) in the pituitary through interaction with corticotropin-releasing hormone receptor 2 (CRHR2; [22]). Similar interactions have been reported in the anterior pituitary of the Western spadefoot toad and the North American bullfrog tadpoles during metamorphosis [23, 24]. Moreover, direct stimulation of CRH has been observed in frogs involved in the acceleration of metamorphosis [25]. Although crosstalk between the stress and thyroid axes has not been demonstrated in fish, it has been reported that *tshba* can be induced by CRH [26]. Considering that CRH is the primary transducer of environmental changes inducing testis development in fish [5], the involvement of *tshba* and their interactions remains unexplored.

## Materials and methods

### Source of medaka

The experiments were performed on medaka (*Oryzias latipes*; strain hi-medaka ID: MT835) supplied by the National BioResource Project (NBRP; www.shigen.nig.ac.jp/medaka/). Fish were maintained and fed following standard protocols for medaka [27] and handled under the ethical regulations of the institutional committee for the Brazilian legislation regulated by the Ethical Principles in Animal Research (Protocol n. 1868-CEUA) and the Institutional Committee for the Care and Use of Experimental Animals from Universidad de San Martín, Argentina (CICUAE-UNSAM 010/2021).

### Sample collection

Sampling was performed at three different stages of development: stage 37, stage 39, and the juvenile-adult stage 20 days post-hatching (dph) [28]. Stage 37 matches the beginning of type II division (cystic proliferation) in the XX gonads; stage 39 corresponds to the maximum embryonic germline stem cells (EGSC) proliferation in XX embryos and involves sexually dimorphic gonads at the exact time of hatching [29]; while at 20 dph, the gonads raise morphological

dimorphisms, which are identified by histological analysis [30]. For all sample collections, fish were euthanized by immersion in tricaine at $30 \pm 50$ mg/L and processed according to the technique used.

## Determination of genotypic sex by PCR

To determine fish genotypic sex, the tail of each animal was treated in 90 µL of 50 mM NaOH at 95 °C for 10 min and equilibrated in 10 µL of 1 M Tris–HCl buffer (pH 8.0). PCR analysis was then performed using primers for [31], and the presence of the β-actin gene was used as a DNA loading control (Supplementary Table S1). PCR conditions were as follows: preheating at 95 °C for 10 min, 40 cycles of PCR at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were analyzed on a 1% agarose gel.

## Total triiodothyronine (T3) levels in fish embryos

T3 levels were measured using an enzyme-linked immunosorbent assay (T3 ELISA kit; EIAT3C, ThermoFisher Scientific). Briefly, embryos at stage 37 were frozen in liquid nitrogen and stored at $-80$ °C. Once PCR determined the sex of each individual, three pools of 50 embryos by treatment and per sex were homogenized in 0.4 mL ELISA buffer using an Ultra-TurraxT8 basic homogenizer (IKA, Staufen, Germany). Then, the structures in the samples were disrupted by intermittent sonic oscillation for 5 min on ice, and the samples vortexed vigorously for 10 min. Samples were then centrifuged for 10 min at $5000 \times g$ at 4 °C. The supernatant was collected and stored at $-80$ °C for T3 assay. Then, 100 µL per reaction was used following the manufacturer's instructions. Each sample was tested in duplicate, and the hormone levels were determined based on a standard curve.

## Gene expression analysis by RT-qPCR

For the TH gene expression profile during the embryonic development of medaka, embryos at stages 37 and 39 were used. The bodies were frozen immediately in liquid nitrogen and stored at $-80$ °C. Once PCR determined the sex of each individual, total RNA was extracted from the five pools of five embryos per treatment, using 500 µL of TRIzol Reagent (Sigma Technologies) following the manufacturer's instructions. The cDNA synthesis was performed with Superscript II (Bio-Rad, CA, USA). Quantitative reverse transcription PCR was performed in a StepOne system (Life Technologies) following the manufacturer's instructions. RT-qPCR reactions were conducted using 5 µL $2 \times$ SYBR-Green Universal Master Mix (Bio-Rad, Hercules, CA, USA), 1 µL of forward primer (9 mM), 1 µL of reverse primer (9 mM), 1 µL of DEPC water, and 2.5 µL of cDNA.

For gene expression of T3 and HT exposure, total RNA was extracted from individual embryos. RNA isolation was carried out using the All Prep DNA/RNA Micro Kit (Qiagen, 80284) following the manufacturer's instructions. The cDNA was synthesized using 200 ng of RNA per sample and the GoScript Reverse Transcription kit protocol with random primers (Promega, Madison, WI, USA) with a Mastercycler Pro S Thermocycler (Thermo Fisher, Ottawa, ON, Canada). The amplification protocol consisted of an initial cycle of 1 min at 95 °C, followed by 10 s at 95 °C and 30 s at 60 °C for a total of 45 cycles.

The subsequent quantification method was performed using the 2-$\Delta\Delta$Ct method (threshold cycle; www.appliedbiosystems.com/support/apptech) and normalized against the reference gene values of β-actin and ribosomal protein L7 (*rpl7*; Zhang and Hu, 2007). Fold change and statistical analysis of RT-qPCR quantifications were performed using the FgStatistics interface (http://sites.google.com/site/fgStatistics/), based on the REST method from Pfaffl et al. [32]. All the primers were validated using a melting curve. The primers were optimized with amplification efficiency between 95 and 105%, a slope of around $-3.30$, and an $R^2$ value greater than 0.99. Primers were designed according to medaka sequences (Supplementary Table S1).

## T3 exposure

Embryos were exposed to 0.5 nM of T3 (Triiodo-L-Thyronine, physiological concentration) and 50 nM (pharmacological concentration) from fertilization until hatching (stage 39) and kept at $24 \pm 0.5$ °C (Control Temperature, CT). The eggs were incubated in 60 mm Petri dishes with embryo medium (17 mM NaCl, 0.4 mM KCl, 0.27 mM CaCl22H2O, and 0.66 mM MgSO4; pH 7) supplemented with each of the concentrations of T3, while the control treatment was with DMSO (0.00325%) as a vehicle for the T3. The medium was renewed every 24 h. For determination of gonadal morphology, after hatching, the larvae were transferred into 2-L water tanks and raised to maturity at a temperature of 25 °C $\pm$ 0.5 °C and constant photoperiod (14L:10D) in a closed-circulation water system as previously established for our laboratory [5].

## Stress thermal treatment

Fertilized medaka eggs were incubated in 60 mm Petri dishes with embryo medium until hatched (stage 39) at a controlled temperature of 24 °C $\pm$ 0.5 °C (control temperature, CT) or 32 °C $\pm$ 0.5 °C (high temperature, HT). Although the standard temperature of housing conditions in medaka ranges from 24–26 °C, this species can adapt to lower (about 4 °C) and higher (37 °C or higher) temperatures (Murata et al., 2019). Furthermore, the temperature of sexual

reversion in medaka was established by Sato et al., [33] and later by Hattori et al., [34], where they observed that from 27ºC onwards, an increase in sexual reversion begins to be seen in this species (10%), with about 50% at 32ºC [27], which was finally the selected temperature. The exposure occurred from fertilization to the stage of interest.

## Histological analysis

Samples for histological examination of gonadal sex ($n = 15$–25 per group) were taken at 20 dph and analyzed following the criteria reported above. Briefly, the animals were anesthetized with 0.1% benzocaine solution, and genotypic sex was determined from the head for all individuals, as previously described. The body trunk was collected and fixed overnight in 2% glutaraldehyde and 4% paraformaldehyde in a Sørensen's phosphate buffer (0.1 M, pH 7.2) or Bouin's solution, sectioned at 5 µm thickness and processed according to standard protocols for preparation of hematoxylin–eosin-stained histological sections. The phenotypic (gonadal) sex of each fish was determined by light microscopic examination (Leica DM6000 BD, Leica Microsystems).

## Generation of double-CRHR (CRHR1 and CRHR2) mutants using CRISPR/Cas9

To generate mutants with two CRH receptors, we used CRISPR/Cas9 following our previous work [5]. Then, mutations of both CRH receptors were selected in F1, and a stable line was generated in F3. Finally, both homozygous mutants were crossed to generate the stable line (KO) in generation F5 (Fig. S1). Finally, the F7 adults were used to obtain fertilized eggs for these experiments. All mutations were analyzed by heteroduplex mobility assay [35] and corroborated by sequencing.

## Immunofluorescence analysis

Medaka embryos at stage 39 were used. All individuals were processed under the same condition for fixation, washing, and incubation with serum and antibodies. The tail was used for sex genotyping by PCR, and the rest of the body was fixed in Bouin's solution overnight. Embryos were then washed with 0.1 M phosphate-buffered saline (PBS, pH 7.4) and blocked in 0.1 M PBS containing 0.5% of bovine serum albumin (Sigma-Aldrich) for 60 min before overnight incubation with a mixture of primary antibody against anti-T4 (rabbit, 1:250; kindly provided by Leandro Miranda, INTECH; [25]) at RT. After incubation, the sections were washed twice in PBS for 10 min each and incubated at RT for 90 min with the secondary antibody goat-anti-rabbit IgG (Life Technologies) combined with Alexa Fluor 488 (green)

at a dilution of 1:2000 in PBS. Separate sets of embryos were treated only with secondary antibodies (negative controls). Photographs of the embryos were taken using the Nikon Eclipse E7000 and the Image Pro Plus (Media Cybernetics) at the same capture conditions of exposure and gain for all samples.

## Treatment with T3, cortisol, methimazole, and glucocorticoid receptor antagonist RU486

This study used inhibitors of THs, methimazole (1-methyl-3H-imidazole-2-thione; Sigma-Aldrich, MO, United States), and a glucocorticoid receptor (Gr) antagonist, mifepristone (RU486, Sigma-Aldrich) to determine whether sex differentiation is affected by THs under thermal stress [36, 37]. Embryos were incubated from 2 h post-fertilization until 5 dph following the recommendations of Hayashi et al. [7]. For the cortisol and T3 synergistic experiment, the embryo medium was complemented with cortisol (5 µM), T3 (0.5 nM), or both (cortisol + T3) at CT $24 \pm 0.5$ °C, with a vehicle of DMSO and ethanol. In the case of rescue, the experiment used 0.15 mM methimazole, 1 µM mifepristone (RU486), and 0.15 mM methimazole combined with 1 µM RU486. As a masculinization control (CHT), each experiment included embryos exposed to 32 °C $\pm 0.5$ °C. The physiological concentration of T3 (0.5 nM) was decided based on results obtained in previous experiments conducted for this research. The concentrations of cortisol, methimazole were selected according to Hayashi et al., [7] and Sharma and Patino [38], respectively. The concentration of RU486 (1 µM) was selected according to Kumai et al., [39], who demonstrated that 1 µM RU486 was able to inhibit the Gr effects in the zebrafish larvae. In addition to its effects on Gr, RU486 can also inactivate the progesterone receptor (Pgr). In this study, we cannot prevent the RU486 effects on the Pgr. However, there is evidence that RU486 is efficient to inhibit the Pgr activity only at higher concentrations. In zebrafish, Chen et al., [40] demonstrated that concentrations higher than 100 µM RU486 were effective to inactivate the Pgr in adult testes. Although we did not perform experiments to evaluate the inactivity of the receptor, the concentration used in our study suggests that the effects reported in our study were due to Gr inhibition and not related to other RU486 targets.

Freshly made dilutions were used, and the treatment solution was changed every 24 h. After the initial exposure, larvae at 5 dph were transferred into 2-L water tanks and raised to maturity at a temperature of 25 °C $\pm 0.5$ °C and constant photoperiod (14L:10D) in a closed-circulation water system until 20 dph, when the genotypic and phenotypic sex was determined as described above. A sample subset was collected at stage 37 for sex genotyping and gene expression profiles following the methods previously described.

## Statistical analysis

All values are represented as mean ± standard error of the mean. Immunohistochemistry quantification was analyzed by $\chi^2$ distribution, and statistical analyses were performed on SPSS v20 software using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences in sex ratio were determined by a hypothesis test for differences in two population proportions, $Z$-test. All differences were considered statistically significant when $p < 0.05$.

## Results

### T3 and thyroid-related genes are up-regulated in males

To investigate the potential role of THs during gonadal sexual differentiation, we first determined the relative expression of thyroid-related genes an T3 level in XX and XY embryos under normal conditions (CT = 24 ºC; Fig. 1A). Our data showed that the T3 level was higher in males than females during the gonadal differentiation period (Fig. 1B). Moreover, as in the activation of thyroid signaling, the thyroid-stimulating hormone receptor (*tshr*) and the iodothyronine deiodinase 2 (*dio2*) involved in the T4 synthesis, secretion and T3 activation, respectively [41, 42], displayed higher expression levels in XY embryos at gonadal differentiation stages (stage 39) than in XX females (Fig. 1C). We also observed that *dio3*, involved in the inactivation of the THs converting T3 to T2 [41], showed higher transcript levels at early stages of gonadal differentiation, stage 37, in XY males than in XX females (Fig. 1C). Moreover, the relative mRNA levels of both thyroid hormone receptors *thra* and *thrb* were up-regulated in XY males than in XX females at stage 37 and 39, respectively (Fig. 1C), reinforcing the participation of the THs in testicular development.

### T3-treatment-induced masculinization of genetic females

Then we performed a second experiment to corroborate the masculinizing effect of THs, in which embryos were maintained in an embryo medium containing two concentrations of T3 (0.5 and 50 nM) from fertilization to hatching (Fig. 2A). Phenotypic analysis demonstrated that both concentrations of T3 induced female-to-male sex reversal; 39% and 27.5% of XX developed testes at 0.5 and 50 nM of T3, respectively (Fig. 2B). No sex reversal was observed in the control group (treated only with vehicle DMSO; Fig. 2B). Taking into account that T3 exposure induced female-to-male reversal, we proceeded to analyze the thyroid-related

genes during the gonadal development period in XX (Fig. 2C) and XY (Fig. S2) embryos. Upstream in the thyroid axis, no differences in thyroid-stimulating hormone (*tshba*) levels were observed in any of the T3 treatments. Further downstream, expression analysis revealed that embryos exposed to 50 nM of T3 displayed significantly higher transcript levels for *dio2* in XX females at stage 39 than in the control group. Interestingly, *dio3* was highly expressed in both sexes only at the highest concentration of T3 treatment (Fig. 2C; Fig. S2). Regarding thyroid hormone signaling, *thra* was almost no change after treatment. At the same time, *thrb* was up-regulated in both XX (Fig. 2C) and XY (Fig. S2) embryos during the gonadal differentiation after treatment with T3 (0.5 and 50 nM).

By analyzing the expression of gonadal sex markers, such as *gsdf* (testicular marker) and *cyp19a1a* (ovarian marker), we found that T3 treatment up-regulated *gsdf* mRNA levels in XX (Fig. 2C), and even in XY (Fig. S2), embryos above those of the control group. On the other hand, *cyp19a1a* was down-regulated in XX embryos treated with T3 (0.5 and 50 nM) compared to control XX embryos (Fig. 2C). In contrast, in XY embryos, it remained low (Fig. S2). This expression of gonadal markers is consistent with the induction of testicular development (Fig. 2B).

### Heat-induced masculinization activates the thyroid axis

To assess the involvement of the thyroid axis in response to heat stress during early gonadal development, the transcript abundance of brain-pituitary-thyroid (BPT) axis-related genes was evaluated in embryos reared at heat (32 °C) and control temperatures (24 ºC; Fig. 3A). Our analysis revealed that heat treatment increased total T3 levels in both XX and XY embryos reared at high temperature (Fig. 3B). Besides, the heat treatment also impacts the expression thyroid-related gene during the gonadal development period in XX (Fig. 3C) and XY (Fig. S3). In agreement with the thyroid axis induction, the expression of *tshba*, the anterior pituitary hormone that stimulates the production and release of T4 from the thyroid follicles [43], increases in both sexes (Fig. 3B; Fig. S3). Moreover, heat treatment affects the expression of deiodinases (*dio2* and *dio3*), showing an up-regulation of *dio2* and a down-regulation of *dio3*, in agreement with the high level of T3 observed. In this regard, *thra* was up-regulated by heat treatment, while *thrb* remained unchanged in XX individuals (Fig. 3C).

Additionally, heat-induced masculinization was confirmed through the expression of testicular and ovarian molecular markers. As previously reported in medaka [5], the testicular marker androgen *gsdf* was up-regulated at the hatching stage in XX embryos reared at a higher

**Fig. 1** Thyroid-related genes and T3 are up-regulated in males during the gonadal differentiation period. **A** Schematic representation of sampling collection during different stages of development (37 and 39) at 24 °C. **B** Quantification of T3 levels in embryos at stage 37 reared at 24 °C. Genotypic sex is represented by XX (full circles) or XY (empty circles). T3 quantification denotes significant differences between groups (*t*-test; $p < 0.05$). **C** Relative expression of thyroid-related genes (*tshr*, *dio2*, *dio3*, *thra* and *thrb*) in embryos at stages 37 and 39 at 24 °C. The *p*-value indicates statistically significant differences between transcript abundance (FgStatistics; $p < 0.05$). *NS* not significant
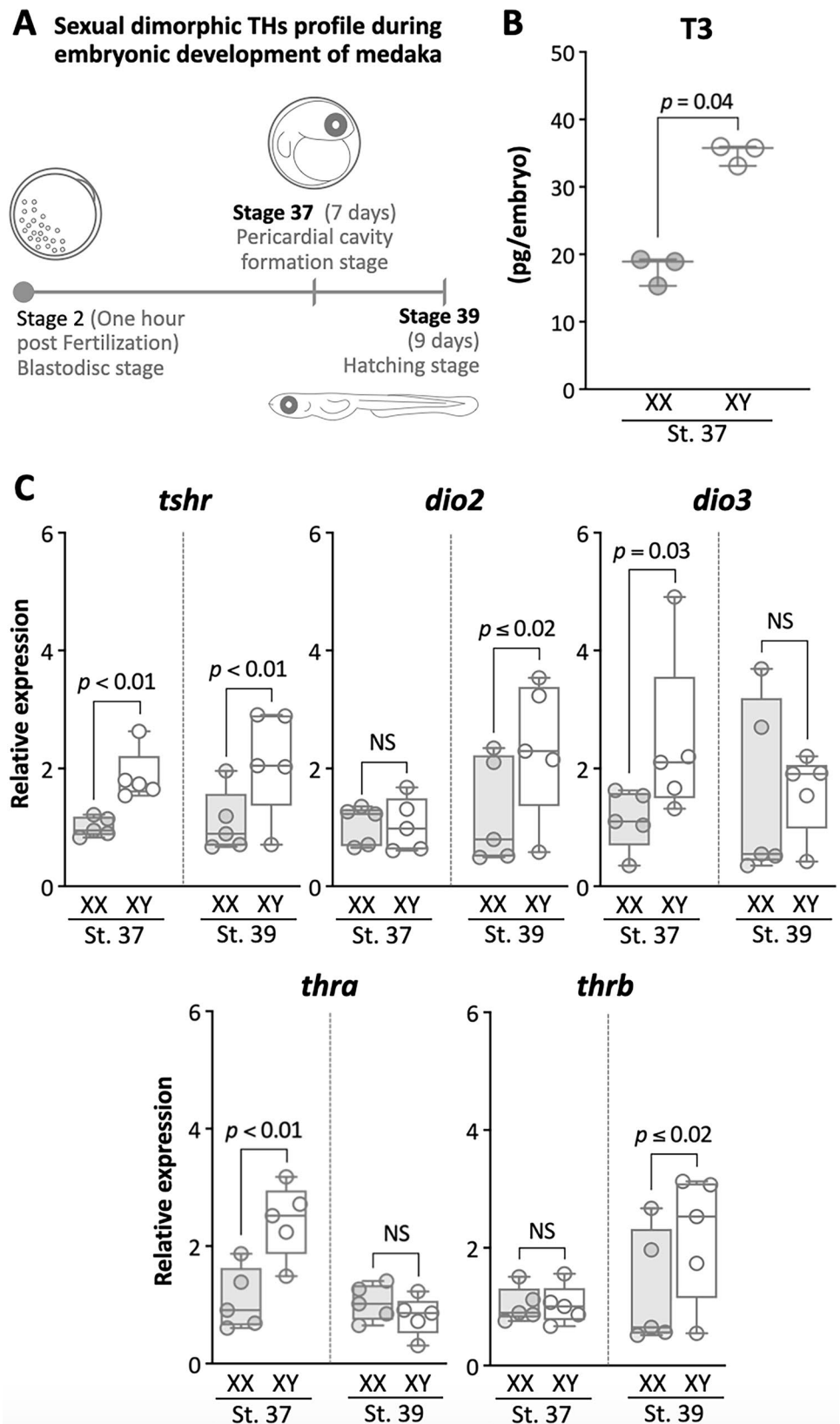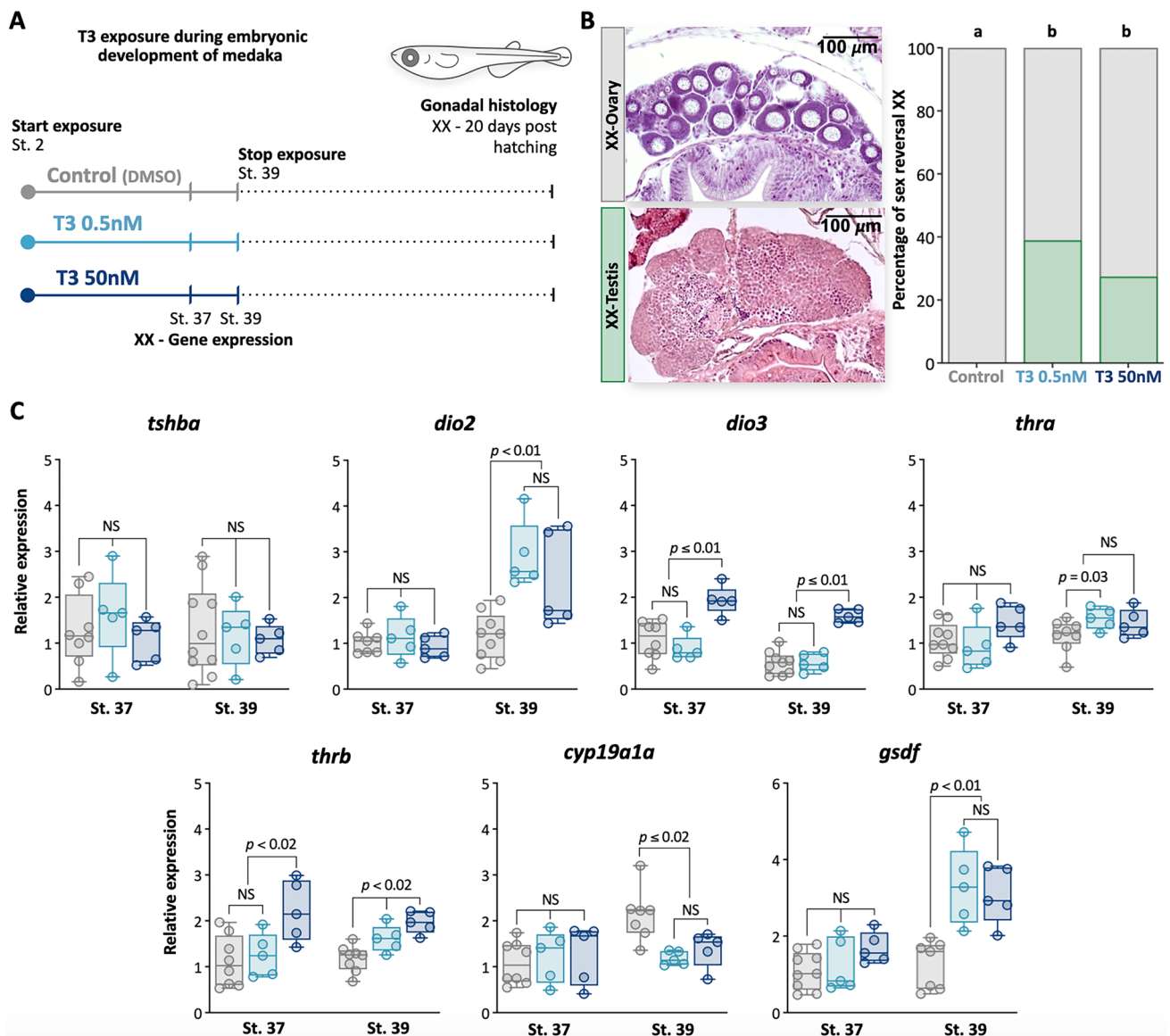
**Fig. 2** T3-treatment-induced masculinization of genetic females. **A** Schematic representation of the T3-treatment experimental procedure. **B** Histological cross sections and percentage of female-to-male reversal individuals (XX Testis) and XX Ovary under T3-treatment at 20 days post-hatching (dph). Staining: Hematoxylin–Eosin; Scale bar = 100 μm. Different letters indicate statistically significant differences between treatments (hypothesis testing to compare two population proportions, Z test, $p < 0.05$). **C** Relative expression of thyroid-related genes (*tshba*, *dio2*, *dio3*, *thra* and *thrb*) and sex differentiation–related genes (*cyp19a1a* and *gsdf*) in XX individuals at 37 and 39 dpf; under treatments of control (DMSO, gray bars), 0.5 nM T3 (light blue bars) and 50 nM T3 (dark blue bars). The *p*-value indicates statistically significant differences between transcript abundance (FgStatistics; $p < 0.05$). *NS* not significant

temperature, while the ovarian marker *cyp19a1a* was downregulated (Fig. 3C), correlating with the masculinizing effects of heat treatment.

## Interaction between thyroid and stress axes during the heat stress-induced masculinization

To assess the interaction between THs and the stress axis in heat-induced masculinization, we analyzed the expression of *tshba* in double-CRHR (CRHR1 and CRHR2) mutant fish (DKO; Fig. 4A). Our data showed a greater expression of *tshba* mRNA in XX embryos reared at a higher temperature than in the XX of the control group (Fig. 4B). On the other hand, these increased levels were nullified in the CRHRs of KO fish exposed to 32 °C, which showed similar levels of *tshba* as those of the wild-type (WT) or KO embryos reared at 24 °C (Fig. 4B).

To corroborate that the loss of function of CRH receptors disrupts the BPT axis, the numbers of thyroid follicles were measured in XX individuals reared at 24 °C
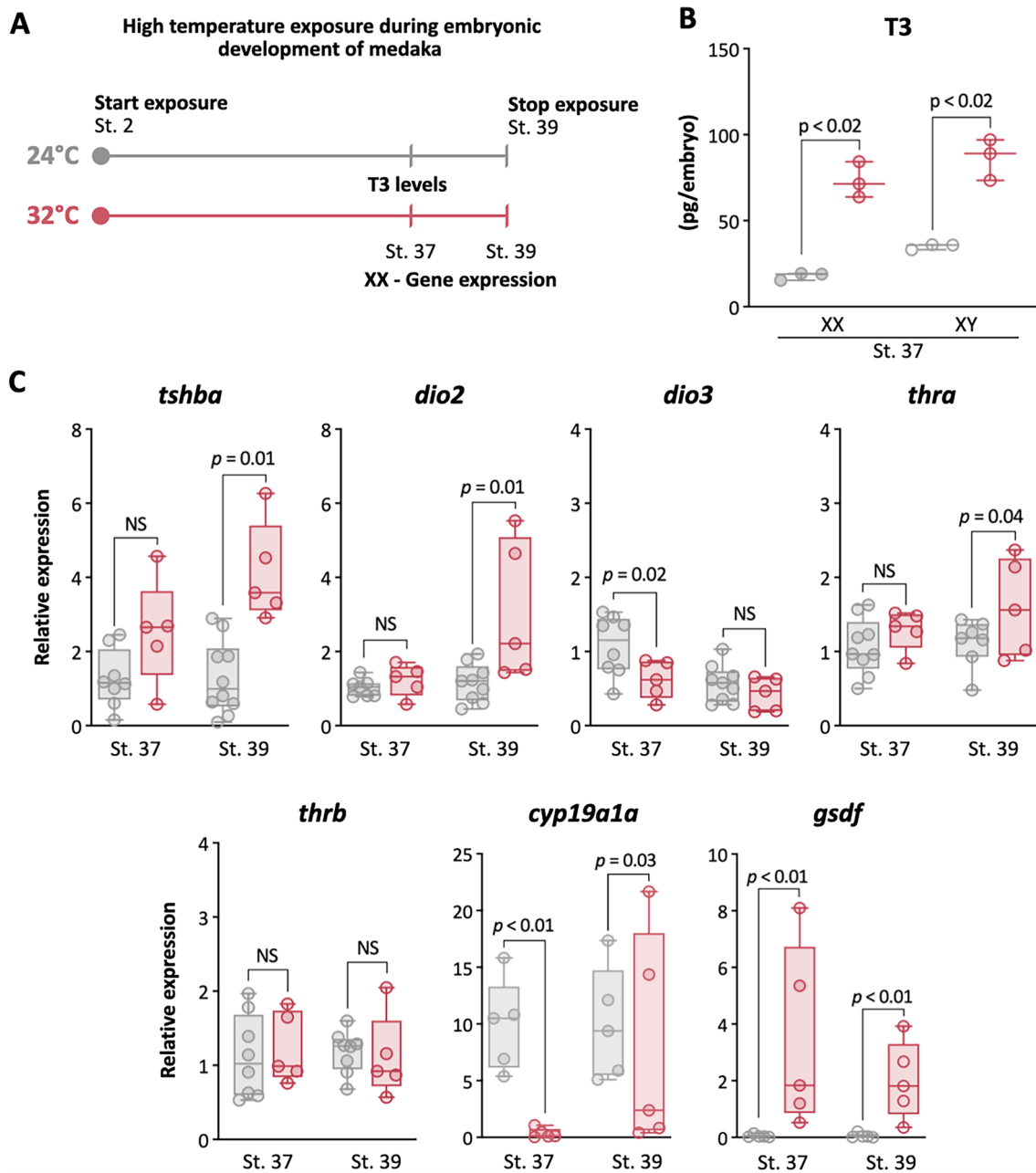
**Fig. 3** Early activation of thyroid hormones and sex differentiation–related genes in response to heat stress. **A** Schematic representation of the thermal stress procedure in embryos at stages 37 and 39 under control (24 °C) and higher temperature (32 °C). **B** Quantification of T3 levels in embryos at stage 37 under control (24 °C) and HT treatment (32 °C). Genotypic sex is represented by XX (full circles) or XY (empty circles). T3 quantification denotes significant differences between groups (*t*-test; *p* < 0.05). **C** Relative expression of thyroid-related genes (*tshba*, *dio2*, *dio3*, *thra* and *thrb*) and sex differentiation–related genes (*cyp19a1a* and *gsdf*) in XX individuals at 37 and 39 dpf under control (24 °C) and HT treatment (32 °C). The *p*-value indicates statistically significant differences between transcript abundance (FgStatistics; *p* < 0.05). *NS* not significant

and 32 °C (Fig. 4C). Analysis of the number of thyroid follicles of XX female embryos raised at both temperatures showed no significant differences (Fig. 4C, D); however, while the number of thyroid follicles showed no differences in CRHR DKOs kept at control temperature, it was significantly lower in CRHR DKOs reared at stressed
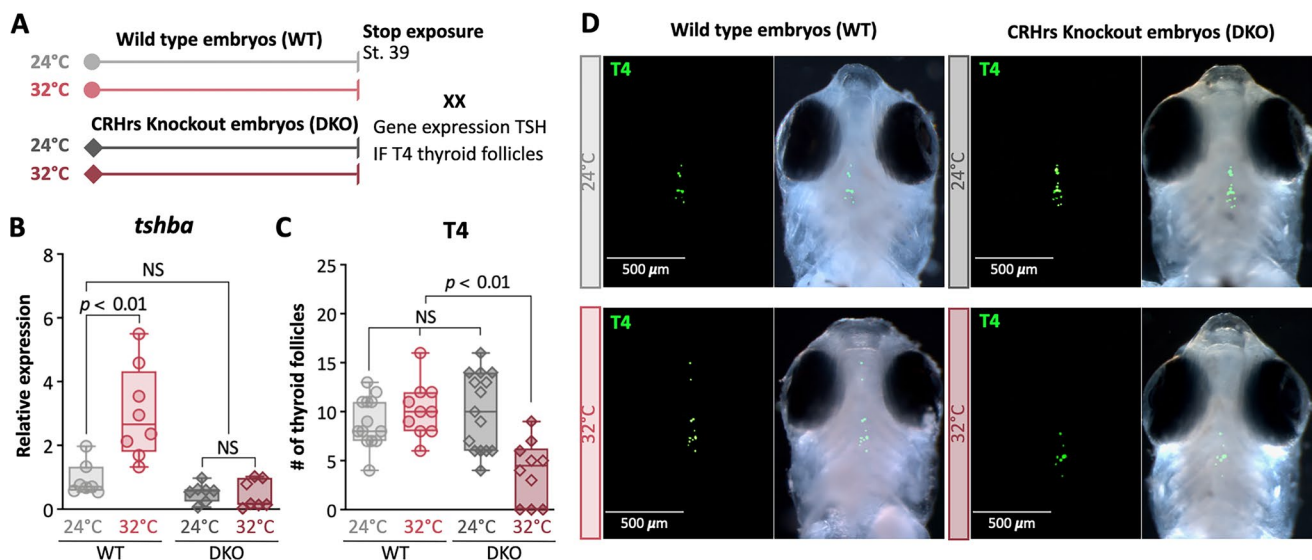
**Fig. 4** CRH receptor knockout reduces the number of thyroid follicles and lacks the up-regulation responses of *tshba* under heat stress. **A** Schematic representation of procedure to evaluate the stress thermal disruption by the double-CRHR (CRHR1 and CRHR2) XX mutants (DKO) at 24 °C (diamonds-dark-gray) and 32 °C (diamonds-dark-red) compared to wild-type individuals at 24 °C (circles-light-gray) and 32 °C (circles-light-red). **B** Relative expression of *tshba, p*-value indicates statistically significant differences between transcript abundance (FgStatistics; $p < 0.05$). *NS* not significant. **C** Numbers of thyroid follicles, *p*-value indicates statistically significant differences between treatments (one-way ANOVA, followed by a Tukey's multiple comparison test; $p < 0.05$). *NS* not significant. **D** Representative fluorescent images of T4 in thyroid follicles in the different treatments (scale bar = 500 μm)

temperature (Fig. 4D), in agreement with the crosstalk between the stress and thyroid axes.

## Thyroid hormones and stress effectors did not have synergistic effects on heat-induced masculinization

Taking into account the crosstalk between stress and thyroid axes (Fig. 4B) and the induction of masculinization in XX medaka by both hormone effectors of each axis, cortisol [7] and T3 (Fig. 2B), we further investigated whether cortisol and T3 could have a synergistic effect on female-to-male sex reversal. Thus, we incubated embryos from 1 dpf to 5 dph in a medium containing cortisol (5 μM), T3 (0.5 nM), or both (cortisol + T3) at 24 ℃ and compared them with control embryos incubated at 32 ℃ (Fig. 5A). Treatments with cortisol (CR), T3, and co-treatment (CR + T3) at 24 ℃ induced female-to-male sex reversal in 38%, 28.5%, and 33% of XX embryos, respectively, without significant differences between treatments (Fig. 5B). However, these sex reversal ratios were significantly lower than the one induced by higher temperature (HT; 52% female-to-male reversal; Fig. 5B).

To rescue the masculinizing effects of cortisol and THs during heat treatment, medaka embryos exposed to a higher temperature (32 ℃) were incubated with a medium containing 0.15 mM methimazole (MM, an inhibitor of THs), 1 μM RU486 (R, a glucocorticoid receptor antagonist) or both (M + R, methimazole + RU486) from 1 dpf to

5 dph (Fig. 5C). Histological analysis showed that pharmacological inhibition of cortisol or thyroid hormone pathways in heat-treated embryos significantly decreased the female-to-male sex reversal compared to the control group (Fig. 5D). The methimazole and RU486 treatments showed female-to-male sex reversal in 16.6% and 8.3% of XX individuals (20 dph), respectively (Fig. 5D). Strikingly, the simultaneous co-inhibition of cortisol and thyroid hormone pathway completely suppressed the female-to-male reversal in XX embryos incubated at the higher temperature (Fig. 5D).

To further investigate the loss of masculinization by the methimazole and RU486 treatments in the temperature stressor treatment, we additionally analyzed molecular markers of ovarian (*cyp19a1a* and *foxl2*) and testicular (*gsdf, amh, amhrII*, and *ar*) development. RT-qPCR analysis revealed that methimazole, RU486, and methimazole + RU486 decreased the transcript levels of *gsdf* in both XX and XY embryos at stage 37 when compared to control (CHT; 32 ℃; Fig. 5E). Similar results were obtained with *amh, amhrII* and *ar* (Fig. S4). Further analysis of female-related genes revealed that cortisol and thyroid hormone inhibitors rescued the female expression of XX individuals reared at higher temperatures (Fig. 5E). *cyp19a1a* and *foxl2* mRNA levels were significantly up-regulated following methimazole, RU486, and methimazole + RU486 treatments in both embryos incubated at 32℃ (Fig. 5E; Fig. S4).

**Fig. 5** Cortisol and T3 participate in the masculinization of XX females without an additive effect. **A** Schematic representation of cortisol (5 µM), T3 (0.5 nM), and both (cortisol + T3) exposure at 24 °C and 32 °C until 39 dpf and sex reversal analysis. **B** Percentage of female-to-male sex reversal in different conditions: NT-control, cortisol, T3, or both (cortisol + T3) at (24 °C), and HT-control (32 °C) at 20 dph. **C** Schematic representation of methimazole (0.15 mM), RU486 (1 µM), and both (methimazole + RU486) exposure at HT (32 °C) until 5 dph and collected at 20 dph for phenotypic sex analysis. **D** Percentage of female-to-male reversal in different conditions: HT-control, methimazole (0.15 mM), RU486 (1 µM), and both (methimazole + RU486) exposure at HT (32 °C). Different letters indicate statistically significant differences between treatments (hypothesis testing to compare two population proportions, Z-test, $p < 0.05$). **E** Relative expression of sex differentiation–related genes (*cyp19a1a and gsdf*) in XX embryos at stage 37 incubated with methimazole (0.15 mM), RU486 (1 µM), or both (methimazole + RU486) at 32 °C. The *p*-value indicates statistically significant differences between transcript abundance (FgStatistics; $p < 0.05$). *NS* not significant
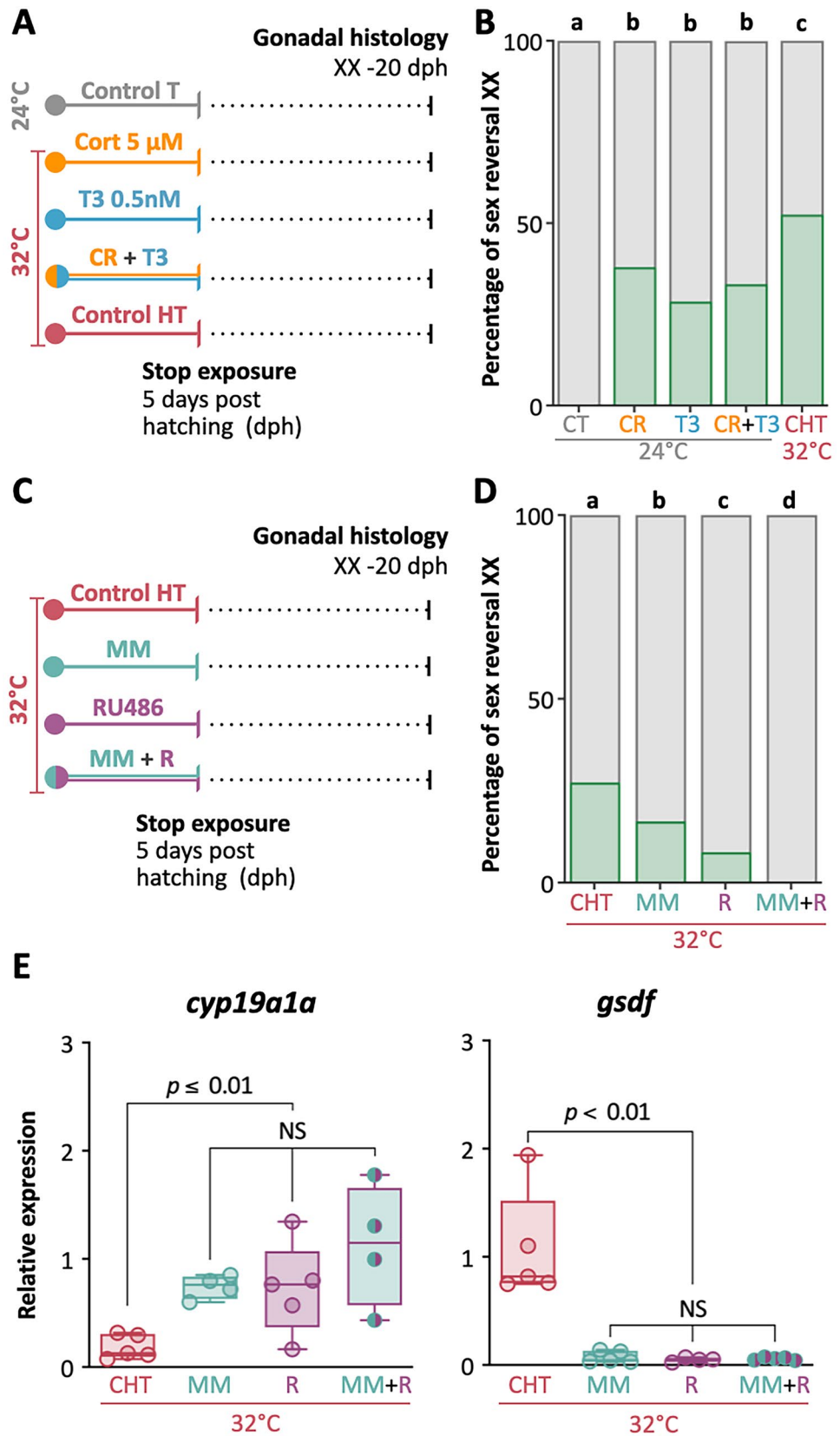
## Discussion

The thyroid and stress axes have been extensively studied individually in relation to testis function (reviewed in [3, 16, 41, 44–46]); however, the interaction between them, particularly on gonadal sex fate, has not yet been elucidated. In the current work, we provided solid evidence that the thyroid axis participates in the female-to-male reversal as a product of the early activation of the stress axis, and both coordinate the direction of the gonadal fate induced by environmental changes.

THs can affect gonadal development and function in vertebrates [44, 46–50]. Several studies have shown that hypothyroidism decreases plasma androgen levels in fish [46, 51, 52], birds [53, 54], and mammals [48, 55, 56]. Furthermore, during early gonadal development in zebrafish, the exposure to perchlorate, an inhibitor of thyroid hormone synthesis, induced a concentration-dependent bias in the sex ratio toward females, and co-treatment with T4 not only blocked the feminizing effect of perchlorate but also biased the sex ratio toward males [18]. In this sense, the masculinizing effect of T4 was later confirmed [19]. Our results with medaka are consistent with this masculinizing effect. Interestingly, increasing the dose of T3 did not increase the levels of sex reversal. This lack of increase in male bias at the higher doses could be due to the fact that T3, like cortisol [12], would act by modulating androgen synthesis, so that at the dose used in this work the threshold for sexual reversal is reached [46, 51, 52], which are fundamental in high-temperature-induced masculinization [13]. We also found that during normal testis development in this species, T3 levels are higher in males, suggesting a novel function of THs in establishing gonadal fate toward testis development.

Another important point we addressed was the crosstalk of the different hormonal axes in heat-induced masculinization. The thyroid, gonadal, and stress axes can interact in diverse organs, and their crosstalk can be established at different levels within each axis [41]. One that we can consider crucial is the evidence that CRH-induced *tshba* is released through direct interaction with CRHR2 in cultured avian pituitary cells [22]. The CRH-induced *tshba* release has also been suggested in frogs [23–25] and fish [26]; however, there is no evidence of the direct interaction between the stress axis and *tshba* in fish. In this regard, we have shown that loss of function of both CRH receptors (CRHR1 and CRHR2) suppresses heat-induced *tshba* expression, with a decreased activation of the thyroid axis, as observed by the reduced number of thyroid follicles. This interaction allows both axes to be activated in the pituitary in response to stress induced by extreme environmental conditions. An exciting observation arises from the analysis of the number of thyroid follicles. A decrease in the number of thyroid follicles was observed due to the loss of function of the CRH receptors. Although the axis still appears to be active, which could be due to adaptive transcriptional compensation or genetic offsets as observed in zebrafish [57], or the classic pathway activation (TRH-TSH-THs) [58] in response to the role of TH in the normal embryonic development of Medaka [59], the significantly lower number of follicles in the CRH receptor double knockout demonstrates the involvement of the stress axis in activating the thyroid axis when environmental changes are perceived as stressful.

In addition to the thyroid-and-stress-axis cross point in the pituitary, cortisol has been reported to promote the conversion of T4 to T3 in adult *Solea senegalensis* and *Salvelinus fontinalis* fish [60, 61], as well as, during larval development in *Sparus aurata* [62]. Furthermore, in vivo studies have shown that corticosteroids contribute to the accumulation of active T3 in target tissues in the tadpole (*Rana catesbeiana)* by inducing the expression of deiodinases involved in T3 synthesis and repressing the expression of deiodinases involved in THs inactivation [63]. Recent studies in the European eel (*Anguilla anguilla*) [64] and in juvenile lake whitefish (*Coregonus clupeaformis*) [65] have reported that THs and outer- and/or inner-ring deiodination may be affected at higher temperatures. Consistent with this, we found that higher temperature increased the expression of activating deiodinases and decreased inhibitory ones, reinforcing higher T3 levels. Interestingly, these high levels of T3 in embryos exposed to high temperatures, which favor masculinization, do not do so with an additive effect on cortisol. In this sense, our results shows that both hormones are essential for the female-to-male sex reversal observed in medaka under heat-stress exposure. Additionally, given that the chemical disruption of both axes under thermal stress does not produce masculinization (methimazole + RU486), these results reaffirm the fact that both axes play a role in this phenomenon.

In summary, four different results support the involvement of the thyroid axis in heat-induced masculinization: (1) T3 levels and expression of genes related to thyroid activation increase during testicular differentiation in XY embryos reared at normal temperature, and XX embryos reared at heat treatment; (2) *tshba* is activated during heat treatment, and the crosstalk between stress and thyroid axes occurs through CRH receptors; (3) the induction of hyperthyroidism causes female-to-male reversal, whereas hypothyroidism suppresses the masculinizing effect of the heat temperature; (4) both hormones, thyroid and cortisol, participate in heat-induced masculinization without an additive effect.

**Data availability** Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

**Ethical approval** Fish were handled under the ethical regulations of the institutional committee for the Brazilian legislation regulated by the Ethical Principles in Animal Research (Protocol n. 1868-CEUA) and the Institutional Committee for the Care and Use of Experimental Animals from Universidad de San Martín, Argentina (CICUAE-UNSAM 010/2021).

**Impact statement** Crosstalk Between the Thyroid and Stress Axes in Thermal-Induced Masculinization in Fish.

## References

1. Capel B (2017) Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nat Rev Genet 18:675–689
2. Ashman TL, Bachtrog D, Blackmon H, Goldberg EE, Hahn MW, Kirkpatrick M, Kitano J, Mank JE, Mayrose I, Ming R, Otto SP, Peichel CL, Pennell MW, Perrin N, Ross L, Valenzuela N, Vamosi JC (2014) Tree of sex: a database of sexual systems. Sci Data 1:140015. https://doi.org/10.1038/sdata.2014.15
3. Hattori RS, Castañeda-Cortés DC, Arias Padilla LF, Strobl-Mazzulla PH, Fernandino JI (2020) Activation of stress response axis as a key process in environment-induced sex plasticity in fish. Cell Mol Life Sci 77:4223–4236. https://doi.org/10.1007/s00018-020-03532-9
4. Dahlke FT, Wohlrab S, Butzin M, Pörtner H-O (2020) Thermal bottlenecks in the life cycle define climate vulnerability of fish. Science 369:65–70. https://doi.org/10.1126/science.aaz3658
5. Castañeda Cortés DC, Arias Padilla LF, Langlois VS, Somoza GM, Fernandino JI (2019) The central nervous system acts as a transducer of stress-induced masculinization through corticotropin-releasing hormone B. Dev 146:1–10. https://doi.org/10.1242/dev.172866
6. Hattori RS, Fernandino JI, Kishil A, Kimura H, Kinno T, Oura M, Somoza GM, Yokota M, Strüssmann CA, Watanabe S (2009) Cortisol-induced masculinization: Does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination? PLoS ONE. https://doi.org/10.1371/journal.pone.0006548
7. Hayashi Y, Kobira H, Yamaguchi T, Shiraishi E, Yazawa T, Hirai T, Kamei Y, Kitano T (2010) High temperature causes masculinization of genetically female medaka by elevation of cortisol. Mol Reprod Dev 77:679–686. https://doi.org/10.1002/mrd.21203
8. Yamaguchi T, Yoshinaga N, Yazawa T, Gen K, Kitano T (2010) Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. Endocrinology 151:3900–3908. https://doi.org/10.1210/en.2010-0228
9. Goikoetxea A, Todd EV, Gemmell NJ (2017) Stress and sex: does cortisol mediate sex change in fish? Reproduction 154:R149–R160. https://doi.org/10.1530/REP-17-0408
10. Nozu R, Nakamura M (2015) Cortisol Administration induces sex change from ovary to testis in the protogynous wrasse, *Halichoeres* trimaculatus. Sex Dev 9:118–124. https://doi.org/10.1159/000373902
11. Chen J, Peng C, Yu Z, Xiao L, Yu Q, Li S, Zhang H, Lin H, Zhang Y (2020) The administration of cortisol induces female-to-male sex change in the protogynous orange-spotted grouper *Epinephelus* coioides. Front Endocrinol (Lausanne) 11:12. https://doi.org/10.3389/fendo.2020.00012
12. Fernandino JI, Hattori RS, Kishii A, Strüssmann CA, Somoza GM (2012) The cortisol and androgen pathways cross talk in high temperature-induced masculinization: the 11β-hydroxysteroid dehydrogenase as a key enzyme. Endocrinology 153:6003–6011. https://doi.org/10.1210/en.2012-1517
13. Castañeda-Cortés DC, Zhang J, Boan AF, Langlois VS, Fernandino JI (2020) High temperature stress response is not sexually dimorphic at the whole-body level and is dependent on androgens to induce sex reversal. Gen Comp Endocrinol 299:113605. https://doi.org/10.1016/j.ygcen.2020.113605
14. Fernandino JI, Hattori RS, Moreno Acosta OD, Strüssmann CA, Somoza GM (2013) Environmental stress-induced testis differentiation: androgen as a by-product of cortisol inactivation. Gen Comp Endocrinol 192:36–44. https://doi.org/10.1016/j.ygcen.2013.05.024
15. Simi S, Peter VS, Peter MCS (2017) Zymosan-induced immune challenge modifies the stress response of hypoxic air-breathing fish (Anabas testudineus Bloch): Evidence for reversed patterns of cortisol and thyroid hormone interaction, differential ion transporter functions and non-specific imm. Gen Comp Endocrinol 251:94–108. https://doi.org/10.1016/j.ygcen.2016.11.009
16. Peter MCS (2011) The role of thyroid hormones in stress response of fish. Gen Comp Endocrinol 172:198–210. https://doi.org/10.1016/j.ygcen.2011.02.023
17. Little AG, Seebacher F (2014) The evolution of endothermy is explained by thyroid hormone-mediated responses to cold in early vertebrates. J Exp Biol 217:1642–1648. https://doi.org/10.1242/jeb.088880
18. Mukhi S, Torres L, Patiño R (2007) Effects of larval-juvenile treatment with perchlorate and co-treatment with thyroxine on zebrafish sex ratios. Gen Comp Endocrinol 150:486–494. https://doi.org/10.1016/j.ygcen.2006.11.013
19. Sharma P, Tang S, Mayer GD, Patiño R (2016) Effects of thyroid endocrine manipulation on sex-related gene expression

and population sex ratios in zebrafish. Gen Comp Endocrinol 235:38–47. https://doi.org/10.1016/j.ygcen.2016.05.028

20. Helmreich DL, Tylee D (2011) Thyroid hormone regulation by stress and behavioral differences in adult male rats. Horm Behav 60:284–291. https://doi.org/10.1016/j.yhbeh.2011.06.003

21. Westphal NJ, Evans RT, Seasholtz AF (2009) Novel expression of type 1 corticotropin-releasing hormone receptor in multiple endocrine cell types in the murine anterior pituitary. Endocrinology 150:260–267. https://doi.org/10.1210/en.2008-0630

22. De Groef B, Goris N, Arckens L, Kühn ER, Darras VM (2003) Corticotropin-releasing hormone (CRH)-induced thyrotropin release is directly mediated through CRH receptor type 2 on thyrotropes. Endocrinology 144:5537–5544. https://doi.org/10.1210/en.2003-0526

23 Denver RJ (1993) Acceleration of anuran amphibian metamorphosis by corticotropin-releasing hormone-like peptides. Gen Comp Endocrinol 91:38–51. https://doi.org/10.1006/gcen.1993.1102

24. Denver RJ (2021) Stress hormones mediate developmental plasticity in vertebrates with complex life cycles. Neurobiol Stress 14:100301. https://doi.org/10.1016/j.ynstr.2021.100301

25. Miranda LA, Affanni JM, Paz DA (2000) Corticotropin-releasing factor accelerates metamorphosis in Bufo arenarum: effect on pituitary ACTH and TSH cells. J Exp Zool 286:473–480. https://doi.org/10.1002/(SICI)1097-010X(20000401)286:5%3c473::AID-JEZ4%3e3.0.CO;2-H

26. Larsen DA, Swanson P, Dickey JT, Rivier J, Dickhoff WW (1998) In vitro thyrotropin-releasing activity of corticotropin-releasing hormone-family peptides in coho salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol 109:276–285. https://doi.org/10.1006/gcen.1997.7031

27. Murata K, Kinoshita M, Naruse K, Tanaka M, Kamei Y (2019) Medaka: biology, management, and experimental protocols. Wiley

28. Iwamatsu T (2004) Stages of normal development in the medaka *Oryzias latipes*. Mech Dev 121:605–618. https://doi.org/10.1016/j.mod.2004.03.012

29. Saito D, Morinaga C, Aoki Y, Nakamura S, Mitani H, Furutani-Seiki M, Kondoh H, Tanaka M (2007) Proliferation of germ cells during gonadal sex differentiation in medaka: Insights from germ cell-depleted mutant zenzai. Dev Biol 310:280–290. https://doi.org/10.1016/j.ydbio.2007.07.039

30. Arias Padilla LF, Castañeda-Cortés DC, Rosa IF, Moreno Acosta OD, Hattori RS, Nóbrega RH, Fernandino JI (2021) Cystic proliferation of germline stem cells is necessary to reproductive success and normal mating behavior in medaka. Elife 10:e62757. https://doi.org/10.7554/eLife.62757

31. Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Schartl M (2002) A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. Proc Natl Acad Sci U S A 99:11778–11783. https://doi.org/10.1073/pnas.182314699

32 Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST ©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:36e. https://doi.org/10.1093/nar/30.9.e36

33. Sato T, Endo T, Yamahira K, Hamaguchi S, Sakaizumi M (2005) Induction of female-to-male sex reversal by high temperature treatment in medaka, *Oryzias latipes*. Zool Sci 22:985–988. https://doi.org/10.2108/zsj.22.985

34. Hattori RS, Gould RJ, Fujioka T, Saito T, Kurita J, Strüssmann CA, Yokota M, Watanabe S (2007) Temperature-dependent sex determination in Hd-rR Medaka Oryzias latipes: gender sensitivity, thermal threshold, critical period, and DMRT1 expression profile. Sex Dev 1:138–146. https://doi.org/10.1159/000100035

35. Ansai S, Kinoshita M (2014) Targeted mutagenesis using CRISPR/Cas system in medaka. Biol Open 3:362–371. https://doi.org/10.1242/bio.20148177

36. Vijayan MM, Reddy PK, Leatherland JF, Moon TW (1994) The effects of cortisol on hepatocyte metabolism in rainbow trout: a study using the steroid analogue RU486. Gen Comp Endocrinol 96:75–84

37. Bandyopadhyay U, Biswas K, Banerjee RK (2002) Extrathyroidal actions of antithyroid thionamides. Toxicol Lett 128:117–127

38. Sharma P, Patiño R (2013) Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish (*Danio rerio*). Gen Comp Endocrinol 184:111–119. https://doi.org/10.1016/j.ygcen.2012.12.018

39. Kumai Y, Nesan D, Vijayan M, Perry S (2012) Cortisol regulates Na+ uptake in zebrafish, *Danio rerio*, larvae via the glucocorticoid receptor. Mol Cell Endocrinol 364:113–125. https://doi.org/10.1016/j.mce.2012.08.017

40. Chen SX, Bogerd J, García-López Á, de Jonge H, de Waal PP, Hong WS, Schulz RW (2010) Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor1. Biol Reprod 82:171–181. https://doi.org/10.1095/biolreprod.109.077644

41. Castañeda Cortés DC, Langlois VS, Fernandino JI (2014) Crossover of the hypothalamic pituitary-adrenal/interrenal, -thyroid, and -gonadal axes in testicular development. Front Endocrinol (Lausanne) 5:139. https://doi.org/10.3389/fendo.2014.00139

42. Chu Y-D, Yeh C-T (2020) The molecular function and clinical role of thyroid stimulating hormone receptor in cancer cells. Cells 9:1730. https://doi.org/10.3390/cells9071730

43. Szkudlinski MW, Fremont V, Ronin C, Weintraub BD (2002) Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. Physiol Rev 82:473–502. https://doi.org/10.1152/physrev.00031.2001

44. Flood DEK, Fernandino JI, Langlois VS (2013) Thyroid hormones in male reproductive development: evidence for direct crosstalk between the androgen and thyroid hormone axes. Gen Comp Endocrinol 192:2–14

45. Tovo-Neto A, da Silva RM, Habibi HR, Nóbrega RH (2018) Thyroid hormone actions on male reproductive system of teleost fish. Gen Comp Endocrinol 265:230–236. https://doi.org/10.1016/j.ygcen.2018.04.023

46. Rodrigues MS, Tovo-Neto A, Rosa IF, Doretto LB, Fallah HP, Habibi HR, Nóbrega RH (2022) Thyroid hormones deficiency impairs male germ cell development: a cross talk between hypothalamic-Pituitary-thyroid, and—gonadal axes in Zebrafish. Front Cell Dev Biol 10:865948. https://doi.org/10.3389/fcell.2022.865948

47. Poppe K, Velkeniers B, Glinoer D (2008) The role of thyroid autoimmunity in fertility and pregnancy. Nat Clin Pract Endocrinol Metab 4:394–405. https://doi.org/10.1038/ncpendmet0846

48. Wagner MS, Wajner SM, Maia AL (2008) The role of thyroid hormone in testicular development and function. J Endocrinol 199:351–365. https://doi.org/10.1677/JOE-08-0218

49. Krassas GE, Poppe K, Glinoer D (2010) Thyroid function and human reproductive health. Endocr Rev 31:702–755

50. Rodrigues MS, Fallah HP, Zanardini M, Malafaia G, Habibi HR, Nóbrega RH (2021) Interaction between thyroid hormones and gonadotropin inhibitory hormone in ex vivo culture of zebrafish testis: An approach to study multifactorial control of spermatogenesis. Mol Cell Endocrinol. https://doi.org/10.1016/j.mce.2021.111331

51. Swapna I, Rajasekhar M, Supriya A, Raghuveer K, Sreenivasulu G, Rasheeda MK, Majumdar KC, Kagawa H, Tanaka H, Dutta-Gupta A, Senthilkumaran B (2006) Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. Comp Biochem Physiol Part

A Mol Integr Physiol 144:1–10. https://doi.org/10.1016/j.cbpa.2006.01.017

52. Morais RDVS, Nóbrega RH, Gómez-González NE, Schmidt R, Bogerd J, França LR, Schulz RW (2013) Thyroid hormone stimulates the proliferation of sertoli cells and single type A spermatogonia in adult zebrafish (*Danio rerio*) testis. Endocrinology 154:4365–4376. https://doi.org/10.1210/en.2013-1308

53. Akhlaghi A, Zamiri MJ (2007) Effect of transient prepubertal hypothyroidism on serum testosterone level and seminal characteristics of chickens. Iran J Vet Res 8:23–31. https://doi.org/10.22099/ijvr.2007.2704

54. Weng Q, Saita E, Watanabe G, Takahashi S, Sedqyar M, Suzuki AK, Taneda S, Taya K (2007) Effect of methimazole-induced hypothyroidism on adrenal and gonadal functions in male Japanese quail (*Coturnix coturnix* japonica). J Reprod Dev. https://doi.org/10.1262/jrd.19081

55. Chiao Y-C, Cho W-L, Wang PS (2002) Inhibition of testosterone production by propylthiouracil in rat leydig cells. Biol Reprod 67:416–422. https://doi.org/10.1095/biolreprod67.2.416

56. Maran RRM (2003) Thyroid hormones: their role in testicular steroidogenesis. Arch Androl 49:375–388. https://doi.org/10.1080/01485010390204968

57. Buglo E, Sarmiento E, Martuscelli NB, Sant DW, Danzi MC, Abrams AJ, Dallman JE, Züchner S (2020) Genetic compensation in a stable slc25a46 mutant zebrafish: a case for using F0 CRISPR mutagenesis to study phenotypes caused by inherited disease. PLoS ONE 15:e0230566

58. Galas L, Raoult E, Tonon M-C, Okada R, Jenks BG, Castaño JP, Kikuyama S, Malagon M, Roubos EW, Vaudry H (2009) TRH acts as a multifunctional hypophysiotropic factor in vertebrates. Gen Comp Endocrinol 164:40–50. https://doi.org/10.1016/j.ygcen.2009.05.003

59. Tagawa M, Hirano T (1991) Effects of thyroid hormone deficiency in eggs on early development of the medaka, *Oryzias latipes*. J Exp Zool 257:360–366. https://doi.org/10.1002/jez.1402570309

60. Arjona FJ, Vargas-Chacoff L, Martín Del Río MP, Flik G, Mancera JM, Klaren PHM (2011) Effects of cortisol and thyroid hormone on peripheral outer ring deiodination and osmoregulatory parameters in the Senegalese sole (*Solea senegalensis*). J Endocrinol 208:323–330. https://doi.org/10.1530/JOE-10-0416

61. Vijayan MM, Flett PA, Leatherland JF (1988) Effect of cortisol on the in vitro hepatic conversion of thyroxine to triiodothyronine in brook charr (*Salvelinus fontinalis* Mitchill). Gen Comp Endocrinol 70:312–318. https://doi.org/10.1016/0016-6480(88)90151-7

62. Szisch V, Papandroulakis N, Fanouraki E, Pavlidis M (2005) Ontogeny of the thyroid hormones and cortisol in the gilthead sea bream, *Sparus aurata*. Gen Comp Endocrinol 142:186–192. https://doi.org/10.1016/j.ygcen.2004.12.013

63. Galton VA (1990) Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone*. Endocrinology 127:2997–3002. https://doi.org/10.1210/endo-127-6-2997

64. Politis SN, Servili A, Mazurais D, Zambonino-Infante J-L, Miest JJ, Tomkiewicz J, Butts IAE (2018) Temperature induced variation in gene expression of thyroid hormone receptors and deiodinases of European eel (*Anguilla anguilla*) larvae. Gen Comp Endocrinol 259:54–65. https://doi.org/10.1016/j.ygcen.2017.11.003

65. Zak MA, Manzon RG (2019) Expression and activity of lipid and oxidative metabolism enzymes following elevated temperature exposure and thyroid hormone manipulation in juvenile lake whitefish (*Coregonus clupeaformis*). Gen Comp Endocrinol 275:51–64. https://doi.org/10.1016/j.ygcen.2019.02.001