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Thyroid axis participates in heat 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 are the leading endocrine players in inducing female-to-male reversal, which can help predict possible upcoming physiological impacts of global warming on fish populations.

Impact Statement

Thyroid and stress axes crosstalk to induce testis development in fish.

Highlights

- Thyroid hormones induce female-to-male reversion.
- Heat temperature induces differentiation of the testes through increased thyroid hormone levels.
- The stress axis, through corticotropin-releasing hormone receptors, promotes thyroid-stimulating hormone expression.
- Both thyroid and cortisol participate in heat-induced fish masculinization.

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 group 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 through increases in temperature changes, and this could negatively impact fish reproduction in the future.

Higher temperatures have been shown to increase plasma cortisol levels in fish [5–7] through activation of the brain-pituitary-interrenal axis [8], which promotes androgen synthesis [9, 10]. Although the

involvement of stress in heat-induced masculinization has been characterized in recent years, the endocrine and molecular processes controlling gonadal 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 [11, 12]. 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 [11–13]. However, the role of THs in stress-induced sex reversal remains unexplored.

Interaction between the thyroid and stress axes has been studied in birds and amphibians. In chickens, corticotropin-releasing hormone (CRH) induced the release of thyroid-stimulating hormone subunit beta a (*tshba*) in the pituitary through interaction with corticotropin-releasing hormone receptor 2 (CRHR2; [14]. Similar interactions have been reported in the anterior pituitary of the Western spadefoot toad and the North American bullfrog tadpoles during metamorphosis [15, 16]. Moreover, direct stimulation of CRH has been observed in frogs involved in the acceleration of metamorphosis [17]. 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 [18]. Considering that CRH is the primary transducer of environmental changes inducing testis development in fish [8], 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 [19] 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) [20]. 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 [21]; while at 20 dph, the gonads raise morphological dimorphisms, which are identified by histological analysis [22]. For all sample collections, fish were euthanized by immersion in tricaine at 30 ± 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 1M Tris-HCl buffer (pH 8.0). PCR analysis was then performed using primers for DMY [23], 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 sec, 59°C for 30 sec, 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×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× 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-ΔΔCt 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. [24]. 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 R2 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 ± 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 ± 0.5°C and constant photoperiod (14L:10D) in a closed-circulation water system as previously established for our laboratory [8].

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 ± 0.5°C (Control Temperature, CT) or 32°C ± 0.5°C (High Temperature, HT). 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 [8]. 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 F7, in which pairs were established to obtain fertilized eggs. All mutations were analyzed by heteroduplex mobility assay [25] 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; [17]) 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 [26, 27]. Embryos were incubated from 2 h post-fertilization until 5 dph following the recommendations of Hayashi et al. [6]. 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 ± 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 ± 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, and RU486 were selected according to Hayashi et al. [6], Sharma and Patino [28], and Kumai et al. [29], respectively. 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 ± 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 χ^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 [30, 31], displayed higher expression levels in XY embryos at gonadal differentiation stages than in XX females (**Fig. 1C**). We also observed that *dio3*, involved in the inactivation of the THs converting T3 to T2 [30], 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 in the evaluated stages of development (**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 HTs, 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; 38% and 32% 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. S1**) 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. Regarding thyroid hormone signaling, *thra* was almost no change after treatment. At the same time, *thrb* was up-regulated in both XX and XY 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. S1**), 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. S1**). 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 ºC) 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. 2C**) and XY (**Fig. S1**). 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 [32], increases in both sexes. 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.

Additionally, heat-induced masculinization was confirmed through the expression of testicular and ovarian molecular markers. As previously reported in medaka [8], the testicular marker androgen *gsdf* was up-regulated at the hatching stage in XX embryos reared at a higher temperature, while the ovarian marker *cyp19a1a* was down-regulated (**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 ºC, which showed similar levels of *tshba* as those of the wild-type (WT) or KO embryos reared at 24 ºC (**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 ºC and 32 ºC (**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 temperature (**Fig. 4D**), in agreement with the over 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 [6] 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 ºC and compared them with control embryos incubated at 32 ºC (**Fig. 5A**). Treatments with cortisol (CR), T3, and co-treatment (CR + T3) at 24 ºC induced female-to-male sex reversal in 38%, 27%, 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 on heat treatment, medaka embryos exposed to a higher temperature (32 ºC) 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 androgen receptor *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 ºC; **Fig. 5E**). Similar results were obtained with *amh, amhrII* and *ar* (**Fig. S3**). 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 32ºC (**Fig. 5E, S3**).

Discussion

The thyroid and stress axes have been extensively studied individually in relation to testis function (reviewed in [3, 12, 30, 33–35]; 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 [33, 35–39]. Several studies have shown that hypothyroidism decreases plasma androgen levels in fish [35, 40, 41], birds [42, 43], and mammals [37, 44, 45]. 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 [46]. In this sense, the masculinizing effect of T4 was later confirmed [47]. Our results with medaka are consistent with this masculinizing effect. 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 [30]. 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 [14]. The CRH-induced *tshba* release has also been suggested in frogs [15–17] and fish [18]; 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 [48], or the classic pathway activation (TRH-TSH-THs) [49] in response to the role of TH in the normal embryonic development of Medaka [50], 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 [51, 52], as well as, during larval development in *Sparus aurata* [53]. 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 [54]. Recent studies in the European eel (*Anguilla anguilla*) [55] and in juvenile lake whitefish (*Coregonus clupeaformis*) [56] 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 iodothyronine deiodinases involved in the activation of THs, such as *dio2*, and down-regulated the inhibitory action of THs, such as *dio3*, in embryos under heat-stress, reinforcing the highest T3 levels observed in the heat-stress exposure compared to XY embryos reared at normal temperature. 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 and; (4) Both hormones, thyroid and cortisol, participate in heat-induced masculinization without an additive effect.

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

dio2: deiodinase type 2

dio3: deiodinase type 3

foxl2: forkhead box protein L2

gsdf: gonadal soma derived factor; testicular marker

MM: Methimazole

R: RU-486; 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

Declarations

CREDIT AUTHOR STATEMENT

DCCC: Conceptualization, Methodology, Visualization and Editing; IFR: Conceptualization, Methodology and Editing; AFB: Methodology; DM: Methodology; NP: Methodology; MAO: Methodology MSR: Methodology; LB: Methodology; CS: Methodology; JTJ: Methodology, DFC: Methodology, SD: Methodology; PHS: Editing; VSL: Conceptualization, Supervision; Writing-Reviewing and Editing; RHN: Conceptualization, Investigation, Supervision, Writing-Reviewing and Editing, JIF: Writing- Original draft preparation, Funding acquisition, Conceptualization, Investigation, Supervision, Writing-Reviewing and Editing.

DISCLOSURE SUMMARY

The authors have no conflict of interest.

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Figures

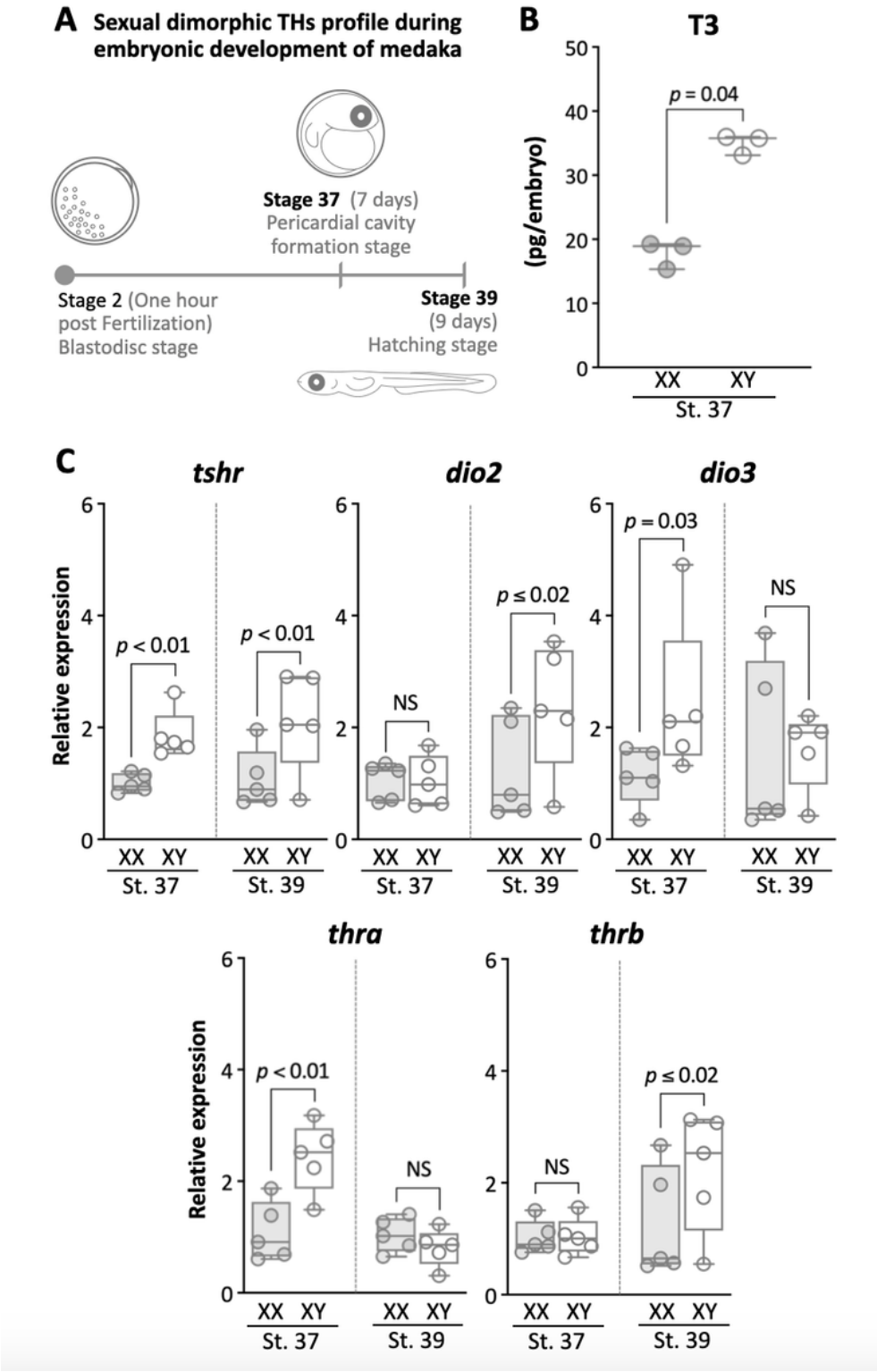

Figure 1

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.

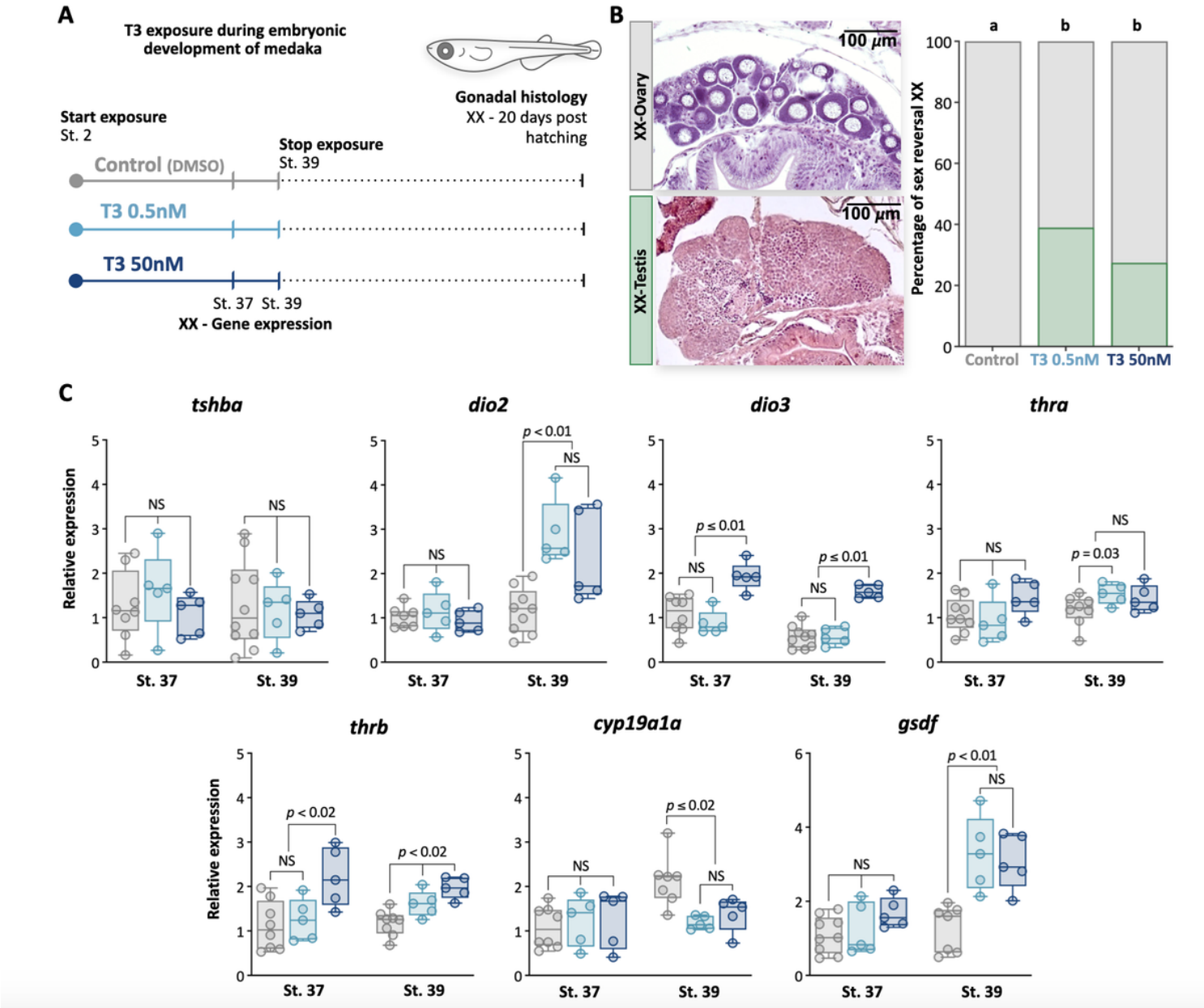

Figure 2

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.

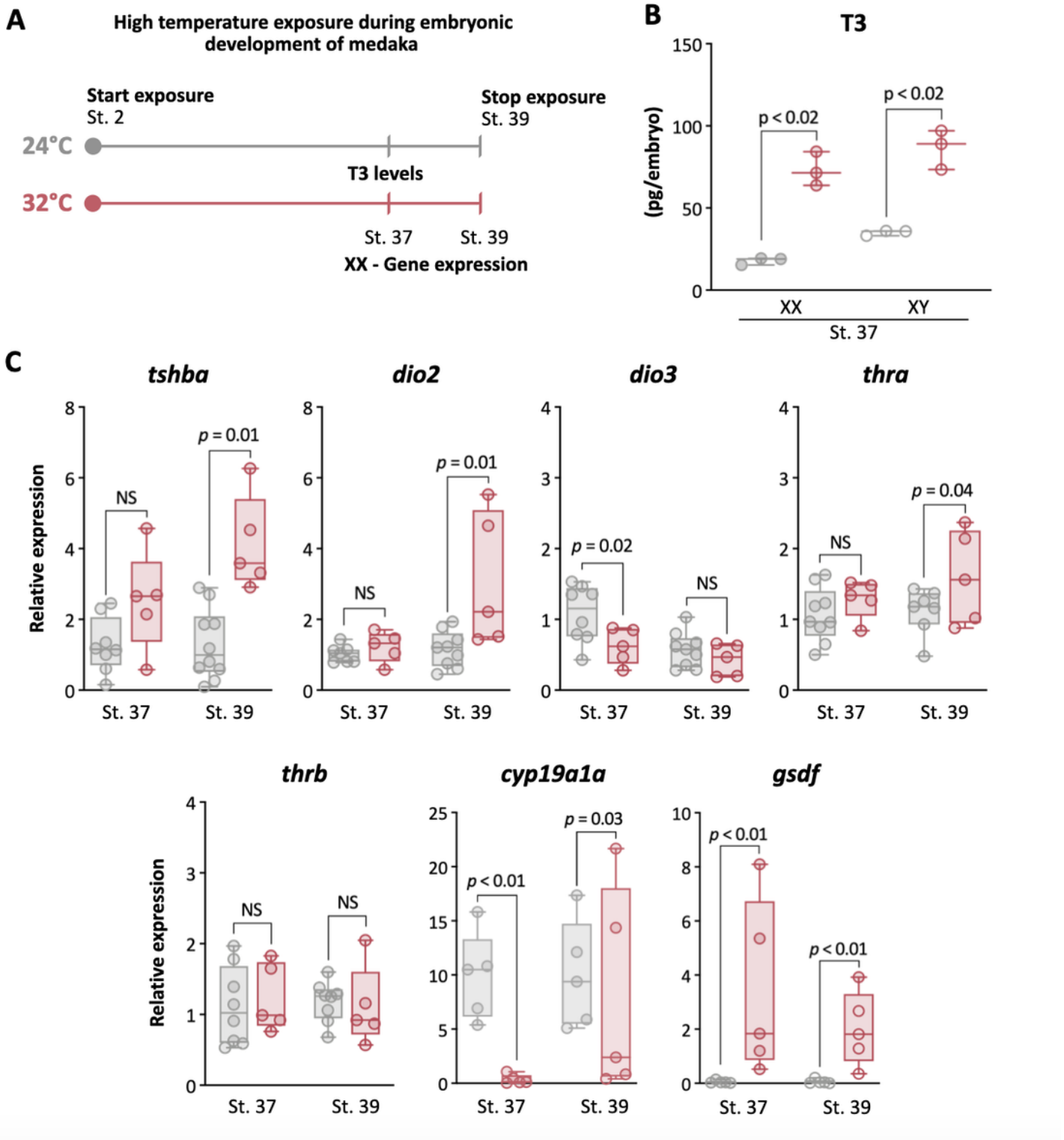

Figure 3

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.

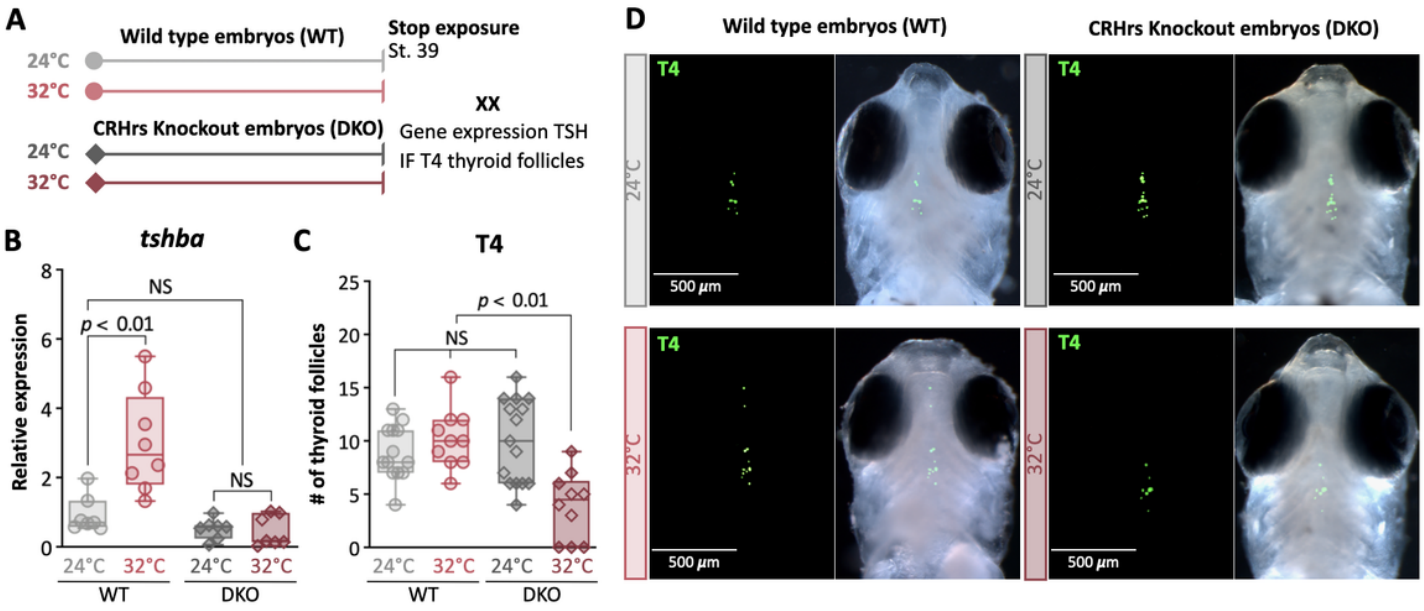

Figure 4

CRH receptor knock-out 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).

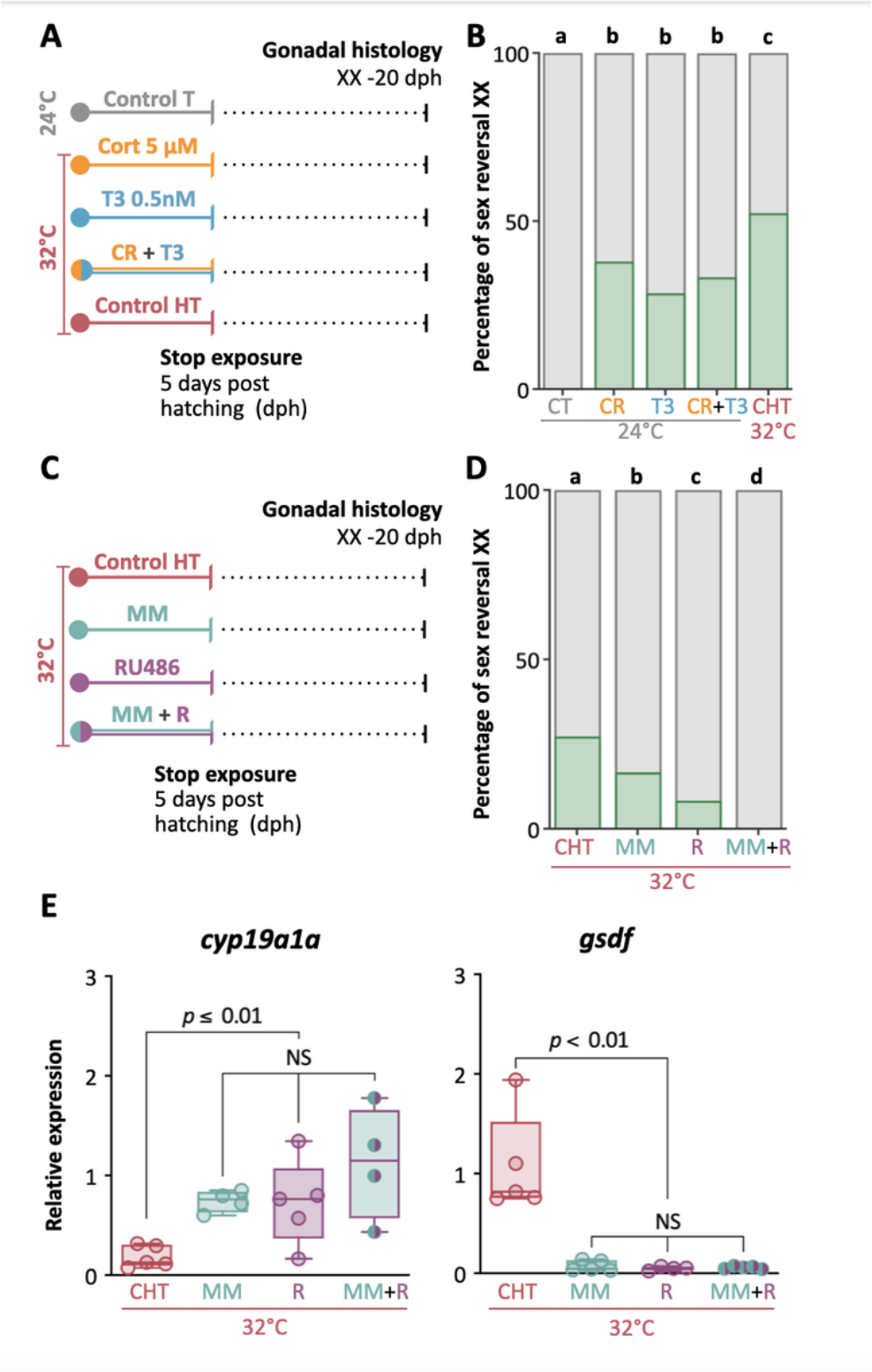

Figure 5

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.

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