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Regulation of thyroid hormones and branchial iodothyronine deiodinases during freshwater acclimation in tilapia

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ABSTRACT

Euryhaline fishes are capable of maintaining osmotic homeostasis in a wide range of environmental salinities. Several pleiotropic hormones, including prolactin, growth hormone, and thyroid hormones (THs) are mediators of salinity acclimation. It is unclear, however, the extent to which THs and the pituitary-thyroid axis promote the adaptive responses of key osmoregulatory organs to freshwater (FW) environments. In the current study, we characterized circulating thyroxine (T4) and 3-3'-5-triiodothyronine (T3) levels in parallel with the outer ring deiodination (ORD) activities of deiodinases (dios) and mRNA expression of *dio1*, *dio2*, and *dio3* in gill during the acclimation of Mozambique tilapia (*Oreochromis mossambicus*) to FW. Tilapia transferred from seawater (SW) to FW exhibited reduced plasma T4 and T3 levels at 6 h. These reductions coincided with an increase in branchial dio2-like activity and decreased branchial *dio1* gene expression. To assess whether dios respond to osmotic conditions and/or systemic signals, gill filaments were exposed to osmolalities ranging from 280 to 450 mOsm/kg in an *in vitro* incubation system. Gene expression of branchial *dio1*, *dio2*, and *dio3* was not directly affected by extracellular osmotic conditions. Lastly, we observed that *dio1* and *dio2* expression was stimulated by thyroid-stimulating hormone in hypophysectomized tilapia, suggesting that branchial TH metabolism is regulated by systemic signals. Our collective findings suggest that THs are involved in the FW acclimation of Mozambique tilapia through their interactions with branchial deiodinases that modulate their activities in a key osmoregulatory organ.

1. Introduction

Euryhaline fishes are capable of tolerating marked changes in environmental salinity such as those that occur during the tidal cycles inherent to coastal areas. To successfully transition between salinities, euryhaline fishes must efficiently control varied physiological processes to maintain osmotic homeostasis. A number of pleiotropic hormones, including prolactin (PRL), growth hormone (GH), cortisol, and thyroid hormones (THs), direct processes central to salinity acclimation (McCormick, 1996; Pickford and Phillips, 1959; Specker et al., 1984). Thyroxine (T4) is the major thyroid hormone secreted by thyroid follicles, with its synthesis primarily regulated by thyroid-stimulating hormone (TSH) released from the pituitary. T4 is further processed by the removal of a single iodine from the outer ring to become 3-3′-5-triiodothyronine (T3), the most biologically active form of TH. Iodine removal is achieved through the activities of iodothyronine deiodinases (dios), and three dio isoforms, denoted dio1, dio2, and dio3, operate in vertebrates (Bianco et al., 2002; Marsili et al., 2011). Outer ring deiodination (ORD) of T4 by dio1 and dio2 results in T3, which upon interacting with nuclear TH receptors elicits an array of biological

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effects through the regulation of gene transcription. On the other hand, inner ring deiodination (IRD) by either dio1 or dio3 results in reverse T3 (rT3), a mostly inactive end-product of TH metabolism (Brent, 2012). By balancing the deiodination activities of three distinct dios, peripheral tissues fine-tune their own responsiveness to TH signaling. Indeed, the capacity for THs to regulate a range of functions, including growth, development, metabolism, and reproduction relies upon the activities of dios (Brent, 2012; Larsen and Zavacki, 2012; Marsili et al., 2011).

Previous studies that investigated the osmoregulatory roles of THs in fish suggested that treatment with T3 or T4 promotes the seawater (SW) tolerance of salmonids (Refstie, 1982; Saunders et al., 1985) and flounder (*Paralichthys dentatus*) (Schreiber and Specker, 1999). In the euryhaline Mozambique tilapia (*Oreochromis mossambicus*), both T3 and T4 increased branchial Na^+/K^+-ATPase (Nka) activity and the size of ionocytes (Peter et al., 2000). Residing in the gill and integument, ionocytes play a central role in systemic osmoregulation by mediating either active ion uptake or extrusion, depending upon the environmental salinity (Kaneko et al., 2008). Accordingly, the exposure of mummichogs (*Fundulus heteroclitus*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*Oncorhynchus mykiss*) to SW increased plasma T4 levels (Knoeppel et al., 1982; Specker and Kobuke, 1987). The SW-induced increase in T4 coincided with decreased renal dio1 and dio2 activities, as shown in rainbow trout (Orozco et al., 2002). Interestingly, plasma T4 levels may also increase in response to a reduction in environmental salinity. For instance, in gilthead sea bream (*Sparus aurata*), T4 levels rose, and branchial dio1 activity dropped, following a change in salinity from 35 to 1 ppt (Klaren et al., 2007). On the other hand, hyposmotic conditions induced hepatic dio2 activity in mummichogs (Lopez-Bojorquez et al., 2007). In striped parrotfish (*Scarus iseri*), hepatic expression of *dio2*, but not *dio1*, was elevated following experimentally-induced hyperthyroidism (Johnson and Lema, 2011). By contrast, dio1 is the primary regulator of circulating T4/T3 levels in mammals (Maia et al., 2011). When considered collectively, these findings suggest that species-specific patterns of thyroid axis regulation are associated with salinity acclimation in fishes. It is also evident that patterns of circulating THs and deiodinase activity have been understudied in paradigms employing hyposmotic challenges (e.g., the transfer of fish from SW to fresh water (FW)).

Because of their capacity to acclimate to a wide range of environmental conditions, tilapiine cichlids continue to be suitable models from which to characterize how various components of the thyroid system operate to support hydromineral balance in fishes. The gene expression and activity of deiodinases show tissue-specific patterns of distribution in tilapia (Mol et al., 1993, 1997; Seale et al., 2014). In Nile tilapia (*O. niloticus*), both *dio1* gene expression and dio1 activity were highest in the kidney (Sanders et al., 1997), while dio2 activity was highest in the liver (Mol et al., 1993). Experimentally-induced reductions of circulating TH levels in Nile tilapia and blackchin tilapia (*Sarotherodon melanotheron*) stimulated hepatic *dio1* and *dio2* gene expression and the activities of their encoded proteins (Van der Geyten et al., 2001). On the other hand, dio3 activity was highest in the brain of Nile tilapia (Sanders et al., 1999), with IRD activity (typical of both dio1 and dio3) also reported in the brain and gill of blue tilapia (*O. aureus*) (Mol et al., 1997). In Mozambique tilapia, *dio1* and *dio3* expression was highest in the gill, while *dio2* was prevalent in both the brain and gill. The branchial expression of all three *dios* was elevated in FW-acclimated fish compared with SW-acclimated fish (Seale et al., 2014); however, these patterns were observed in fish permanently acclimated to steady-state conditions and may not reflect *dio* expression patterns when fish are experiencing changes in salinity. The mummichog *dio2* gene contains two functional osmotic response elements in its promoter (Lopez-Bojorquez et al., 2007), further implicating dios in mediating adaptive responses to changes in salinity.

Although deiodinases have been identified as key players in the tissue-level regulation of TH availability during salinity challenges (Orozco and Valverde, 2005), the role of branchial deiodinases, especially during a hyposmotic challenge, remains unclear. Given that hyperosmotic challenges (e.g., FW to SW transfer) generally increase plasma T4 levels in euryhaline teleosts (Dickhoff et al., 1978; Orozco et al., 2002; Peyghan et al., 2013; Specker and Kobuke, 1987), we hypothesized that a hyposmotic challenge would evoke the opposite response in Mozambique tilapia. Moreover, because TH metabolism is fine-tuned by dios in peripheral tissues, we sought to further resolve how branchial dio activity and expression may support adaptive responses to salinity transitions. In the current study, we transferred SW-acclimated tilapia to FW and characterized attendant patterns of plasma T3 and T4, branchial dio activity, and branchial *dio* mRNA levels. We also employed a gill filament incubation system to probe the local regulation of *dio* expression by extracellular osmotic conditions, and hypophysectomy with TSH replacement to investigate the systemic regulation of branchial *dio* expression.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified.

2.2. Animals

Mature Mozambique tilapia of both sexes were obtained from a population maintained at the Hawai'i Institute of Marine Biology, University of Hawai'i. Fish were reared in outdoor tanks (700 L) with a continuous flow of FW (municipal water) or SW (Kaneohe Bay, Hawai'i, USA) under natural photoperiod. SW-acclimated tilapia employed in this experiment were spawned, and continuously reared in SW, having never been previously exposed to FW. Animals were fed ~5% of their body weight per day with Trout Chow (Skretting, Tooele, UT). All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawai'i.

2.3. Salinity transfer experiments

Two separate salinity-transfer experiments were performed with fish of both sexes. In both experiments, at the time of transfer, tanks were transitioned from SW (34 ppt) to FW (0 ppt) with minimal disturbance by opening an incoming FW-valve and closing a SW-valve. FW conditions were reached after 60 min. Time-matched control tanks, which contained fish from the original pool raised in SW, were held at constant salinity throughout the duration of the experiment. Fish were fed for the duration of the experiment and water temperature was maintained between 24 and 26 °C. In the first experiment, fish weighing 41.4 ± 1.4 g (mean ± S.E.M.) were sampled (n = 6–10) at 0 h (immediately prior to opening the FW-valve), 6 h, 48 h, and 10 d after transfer and gill filaments were collected for deiodinase activity assays. In the second experiment, fish weighing 362.7 ± 23.1 g were sampled (n = 8) at 0 h, 6 h, 24 h, 48 h, and 7 d after transfer, and blood plasma samples were used for the assessment of plasma osmolality and THs. Gill filaments were also collected for *dio* gene expression analyses. Fish were fasted for 24 h prior to sample collection. At the time of sampling, fish were netted and anesthetized with 2-phenoxyethenol (2-PE; 0.3 mL/L). Blood from the caudal vasculature was collected using a syringe coated with heparin ammonium salt (200 U/mL). Anesthetized fish were euthanized by rapid decapitation prior to the collection of gill filaments. All tissue samples were stored at −80 °C prior to their analysis.

2.4. Plasma osmolality and thyroid hormones

Plasma was collected following the centrifugation of blood samples (10,000 rpm for 10 min). Plasma osmolality was measured using a vapor

pressure osmometer (Wescor 5100C, Logan, UT, USA). Two 25 μL volumes of plasma were used to assay THs using the AccuDiag™ ELISA – T4 kit (Diagnostic Automation; Woodland Hills, CA) and the T3 (Total) ELISA kit (Abnova; Taipei, Taiwan) according to the manufacturers' instructions. Both assays were validated using stripped tilapia plasma following overnight incubation with 20 mg/mL of dextran-coated charcoal at 4 °C. Stripped tilapia plasma was spiked with known concentrations of T4 or T3. Stock solutions of THs were made in 0.01 N sodium hydroxide and diluted in phosphate buffer. The standard curves obtained with stripped tilapia plasma showed parallelism with those generated by commercial kits (Supplementary Fig. 1). Intra-assay coefficients of variation were 6.5% and 5.6% for T4 and T3, respectively, while inter-assay coefficients of variation were 9.6% and 1.8% for T4 and T3, respectively.

2.5. Deiodinase activity assays

Deiodinase assays were performed as previously described (Mol et al., 1997; Steinsapir et al., 1998; Zavacki et al., 2005). Briefly, gill filaments were sonicated in chilled 0.1 M potassium phosphate buffer (pH 6.9) containing 1 mM EDTA (PE buffer), 0.25 M sucrose, 10 mM dithiothreitol (DTT), and a protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentration was determined using the colorimetric Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) with a bovine serum albumin (BSA) standard curve. Freshly Sephadex-purified outer ring labeled [I^{125}]-T4 averaged ~109,790 counts per min (cpm; PerkinElmer, Waltham, MA). For each sample, 100 μg of protein was incubated overnight at 37 °C in PE buffer containing 20 mM DTT, ~80,000 cpm of outer ring labeled [I^{125}]-T4, and either 0.5 nM or 100 nM of cold T4, in a total reaction volume of 110 μl. BSA (100 μg) was used as background (~2623 cpm) under the same reaction conditions as above, and subtracted from experimental sample counts.

Deiodination was stopped by precipitation with 67 μl of horse serum (Gibco® Thermo Fisher Scientific, Waltham, MA) and 33 μl 50% trichloroacetic acid, followed by vortexing and centrifugation (12,000 rpm for 3 min). 120 μl of supernatant was counted with a Packard Cobra gamma-counter (PerkinElmer). The activity from purified human Dio2 (hDio2) was used to represent total activity, for subsequent calculations, based on its obligate outer ring deiodination activity (Bianco et al., 2002). hDio2 was immunopurified after transient transfection of N-terminal peptide FLAG-tagged plasmid construct of hDio2 cDNA (NM_013989.5) overexpressed in a human embryonic kidney (HEK-293) cell line. Transient transfection was carried out by the calcium phosphate method for 48 h (Brent et al., 1989). Total deiodination was calculated based on the cpm of samples when incubated with 0.5 nM cold T4. High Km T4 ORD activity was calculated by subtracting blank counts from the counts obtained with 100 nM cold T4. Low Km T4 ORD activity was calculated by subtracting counts obtained from samples incubated with 0.5 nM cold T4 from counts obtained with samples incubated with 100 nM cold T4. For these calculations, we used the approach and nomenclature suggested previously (Mol et al., 1997) regarding the kinetic characteristics of the ORD enzymes. Specifically, considering that some dio1 activity cannot be excluded from counts at 0.5 nM T4, and some dio2 activity cannot be excluded from counts at 100 nM T4, the high and low Km T4 ORD activities are referred to as dio1-like and dio2-like, respectively. All results were calculated by expressing the cpm values as a percentage of the counts obtained with the hDio2 sample.

2.6. Gill filament incubation

To assess whether extracellular osmolality can directly affect branchial *dio* transcript levels, we incubated filaments from the second and third gill arches of male FW-acclimated tilapia (~100 g) following a previously described protocol (Watanabe et al., 2016) and experimental design (Inokuchi et al., 2015). Briefly, excised gill arches were first washed in sterilized balanced salt solution (BSS: NaCl 120 mM; KCl 4.0 mM; $MgSO_4$ 0.8 mM; $MgCl_2$ 1.0 mM; $NaHCO_3$ 2.0 mM; $CaCl_2$ 1.5 mM; KH_2PO_4 0.4 mM; Na_2HPO_4 1.3 mM; $CaCl_2$ 2.1 mM; Hepes 10 mM; pH 7.4) and then incubated in 0.025% $KMnO_4$ for 1 min. After a second wash in BSS, individual gill filaments were cut from the arches, cut sagittally under a dissecting microscope, and then placed (3 filaments/well) in 24-well plates (Becton-Dickinson, Franklin Lakes, NJ) containing sterilized isosmotic (330 mOsm/kg) Leibovitz's L-15 culture medium (Gibco® - Thermo Fisher Scientific) supplemented with 5.99 mg/L penicillin and 100 mg/L streptomycin. Filaments were incubated in 500 μL of incubation medium adjusted to four different osmolalities, 280, 330, 380, and 450 mOsm/kg (n = 8), which reflect the range of plasma osmolalities encountered by Mozambique tilapia following abrupt salinity changes (Seale et al., 2012a). The hyposmotic medium (280 mOsm/kg) was produced by diluting incubation medium with distilled water. Isosmotic (330 mOsm/kg) and hyperosmotic (380 and 450 mOsm/kg) media were produced by adding 5 moL/l NaCl solution to the hyposmotic medium. Osmolalities of incubation media were verified using a vapor pressure osmometer (Wescor 5520, Logan, UT). After incubation for 3 and 6 h at 26 °C, gill filaments were frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction and gene expression analyses.

2.7. Hypophysectomy and TSH replacement

Hypophysectomy was performed by the transorbital technique described by (Nishioka, 1994). Prior to surgery, male FW-acclimated fish (118.4 ± 6.9 g) were anesthetized in buffered tricaine methanesulfonate (100 mg/L, Argent Chemical Laboratories, Redmond, WA) and 2-PE (0.3 mL/L) in FW. After the procedure, fish recovered in experimental aquaria containing recirculating brackish water (12 ppt; 24–26 °C) where they were maintained for 3 days. Fish were treated with kanamycin sulfate (National Fish Pharmaceuticals, Tucson, AZ) and not fed following surgery. Three days after hypophysectomy, fish (n = 6–9) were anesthetized with 2-PE (0.3 mL/L) and administered bovine TSH (bTSH; 5 μg/g body weight or 0.01 IU/g body weight) or saline vehicle (0.9% NaCl) by a single intraperitoneal injection (1.0 μl/g body weight). All animals were treated in the same fashion prior to injections. To our knowledge, this is the first study that injected hypophysectomized tilapia with bTSH; the concentration of bTSH administered was based on previous hypophysectomy and hormone replacement studies in this species employing heterologous hormones such as ovine PRL and ovine GH (Breves et al., 2010b, 2014). After injection, fish were returned to the experimental aquaria and sampled after 12 h. At the time of sampling, fish were euthanized and plasma and gill filaments (from the second left arch) were collected. Samples were snap frozen in liquid nitrogen and stored at −80 °C.

Table 1
Specific primer sequences (5′–3′) for quantitative real-time PCR.

Gene	Forward	Reverse	Reference
dio1	AACTATGAGGATTGGGGTCT	TGAGTCTGGAGCTTCTCCT	Seale et al. (2014)
dio2	CTTCTGTTTGTGCGTTTACA	TTCCAAACACTTTTCTCGTT	Seale et al. (2014)
dio3	AGAAACTGGCTGGAACAATA	ATGGGTGAACATCTGATAGC	Seale et al. (2014)
ef1a	AGCAAGTACTACGTGACCATCATTG	AGTCAGCCTGGGAGGTACCA	Breves et al. (2010a)

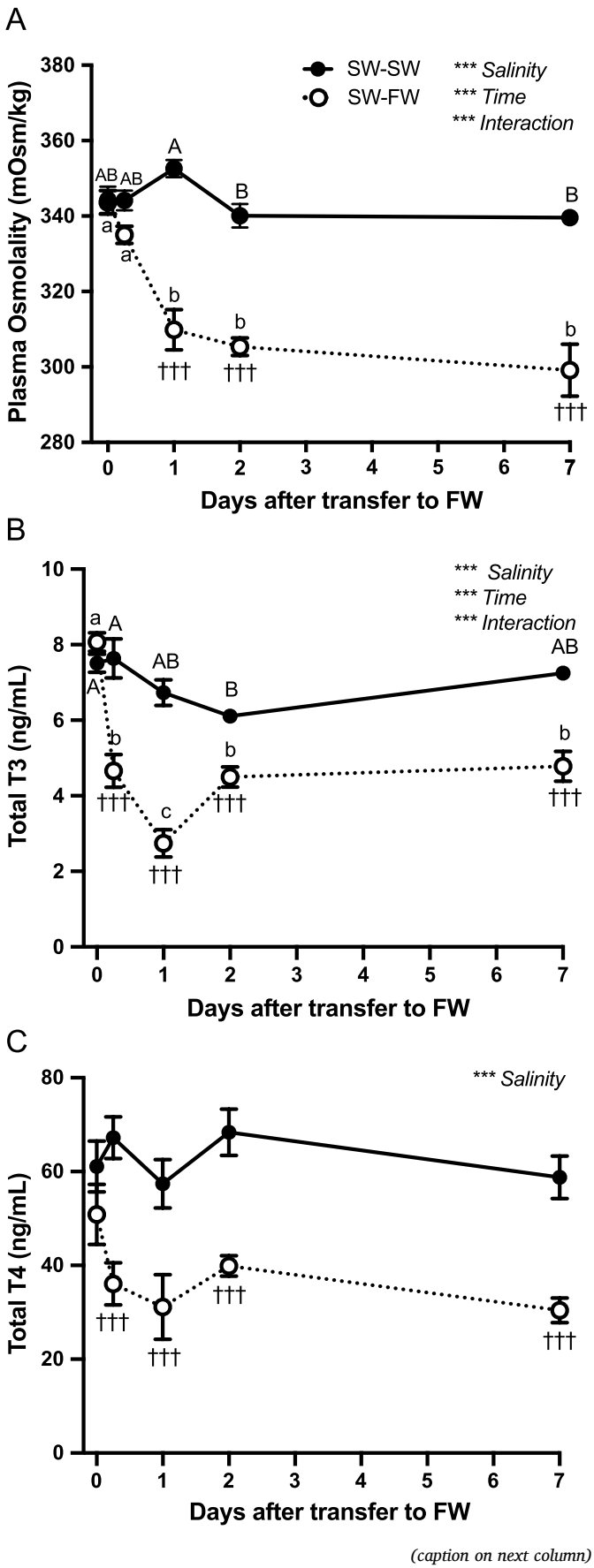

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Fig. 1. Changes in plasma osmolality (A), T4 (B), and T3 (C) after transfer of Mozambique tilapia from SW to FW (open circles). Symbols represent mean ± S.E.M ($n = 7$–8). Control fish were maintained in SW (solid circles) and sampled on the same time course as fish subjected to a FW challenge. Differences among groups were evaluated by two-way ANOVA. Main and interaction effects are marked with *** representing $P < 0.001$. Significant ($P < 0.05$) main and interaction effects (marked with asterisks) were followed up by Fisher's protected LSD test. †, ††, ††† denote significant differences from corresponding SW–SW controls at $P < 0.05$, 0.01, and 0.001, respectively, and different letters correspond to significant differences in means across time within SW-FW (lower case) and SW-SW (upper case) groups.

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRI Reagent according to the manufacturer's protocols (MRC, Cincinnati, OH). The concentration and purity of extracted RNA were assessed using a NanoDrop (NanoDrop One, Thermo Fisher Scientific, Waltham, MA). Total RNA (400 ng) was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The mRNA levels of reference and target genes were determined by the relative quantification method using a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The qPCR reaction mix (15 µl) contained Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 200 nM of forward and reverse primers, and 1 µl cDNA. PCR cycling parameters were: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All gene specific primer sequences are provided in Table 1. After verification that elongation factor 1α (*ef1a*) mRNA levels did not exhibit significant main effects (salinity: $P = 0.73$; time: $P = 0.11$), *ef1a* levels were used to normalize *dio* gene expression levels. Reference and target genes were calculated by the relative quantification method with PCR efficiency correction (Pfaffl, 2001). Standard curves were prepared from serial dilutions of gill cDNA and included on each plate to calculate the PCR efficiencies for target and normalization gene assays. Relative gene expression ratios between groups are reported as a fold-change from controls.

2.9. Statistical analyses

Data from salinity transfer experiments were analyzed by two-way analysis of variance (ANOVA) with salinity and time as main effects. Significant ($P < 0.05$) main and interaction effects (marked with asterisks) were followed up by Fisher's protected LSD test. In gill filament incubation experiments, data are expressed as percent change from isosmotic (330 mOsm/kg) controls and slope analyses were carried out by linear regression. The effects of hypophysectomy and hormone replacement on branchial *dios* were analyzed by one-way ANOVA followed by Fisher's protected LSD test. Statistical calculations were performed using Prism v. 9 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Effects of salinity on plasma osmolality and circulating thyroid hormones

It is well-established that plasma osmolality drops upon transfer of tilapia from SW to FW (Seale et al., 2002). In the transfer paradigm used in the current study, we found a significant reduction in plasma osmolality, from ~340 to ~300 mOsm/kg, at 24 h after transfer to FW; this reduction was sustained until the end of the experiment on day 7. In SW-SW controls, plasma osmolality was maintained at ~340 mOsm/kg throughout the experiment (Fig. 1A). Transfer from SW to FW led to rapid decreases in both plasma T4 and T3 (Fig. 1B and C) by 6 h. T4 levels fell from 50 to 60 ng/mL to 30–40 ng/mL and remained at the lower levels for the duration of the experiment. T3 levels, on the other hand, fell from ~8 ng/mL to ~5 ng/mL by 6 h, dipped further to ~3

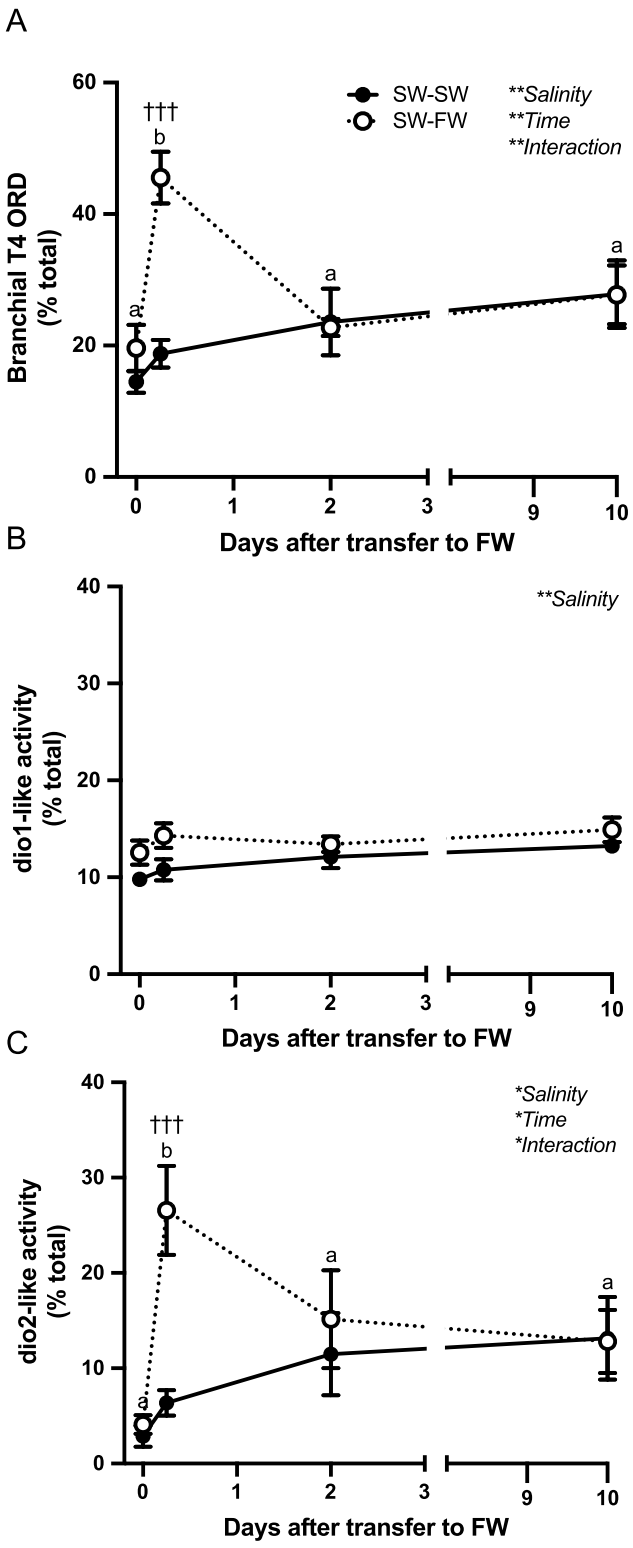


Fig. 2. Branchial T4 ORD (A), dio1-like (B), and dio2-like (C) activities after transfer of Mozambique tilapia from SW to FW (open circles). Dio1-like and dio2-like correspond to high and low Km activities, respectively. Symbols represent mean ± S.E.M (n = 5). Control fish were maintained in SW (solid circles) and sampled on the same time course as fish subjected to a FW challenge. Differences among groups were evaluated by two-way ANOVA. Main and interaction effects are marked with *, ** representing $P < 0.05$, and 0.01, respectively. Significant effects between means were followed up by Fisher's protected LSD test. ††† denotes significant differences from corresponding SW–SW controls at $P < 0.001$, and different letters correspond to significant differences in means across time within SW-FW (lower case) groups.

ng/mL by 24 h, and then leveled at ~4.5 ng/mL for the remainder of the experiment. SW-SW controls maintained circulating levels of T4 and T3 at ~60–70 ng/mL and ~6–8 ng/mL, respectively.

3.2. Effects of salinity on branchial deiodination activity in vivo

Fish transferred from SW to FW exhibited a doubling of total branchial T4 ORD activity at 6 h. This increase, however, was transient, with activity returning to baseline levels by 48 h (Fig. 2A). Despite a significant main effect of salinity on a high Km T4 ORD activity, no differences between groups at any individual time points were detected by *post hoc* analyses (Fig. 2B). Resembling the pattern of total branchial T4 ORD activity, low Km T4 ORD activity was markedly increased in the SW-FW treatment compared with SW-SW controls at 6 h (Fig. 2C).

3.3. Effects of salinity on deiodinase gene expression in vivo

We then investigated the branchial gene expression of *dios* following the transfer of tilapia from SW to FW. There was a significant main effect of time on *dio1* expression (Fig. 3A). *dio1* expression was transiently reduced at 6 h after transfer from SW to FW and then gradually rose to initial levels by 7 d. There was a significant interaction effect, but no main effects, detected for *dio2* expression. A transient increase in *dio2* occurred by 6 h in the SW-SW control group (Fig. 3B). There were no significant main effects or an interaction for branchial *dio3* expression (Fig. 3C).

3.4. Effects of extracellular osmolality on deiodinase gene expression in vitro

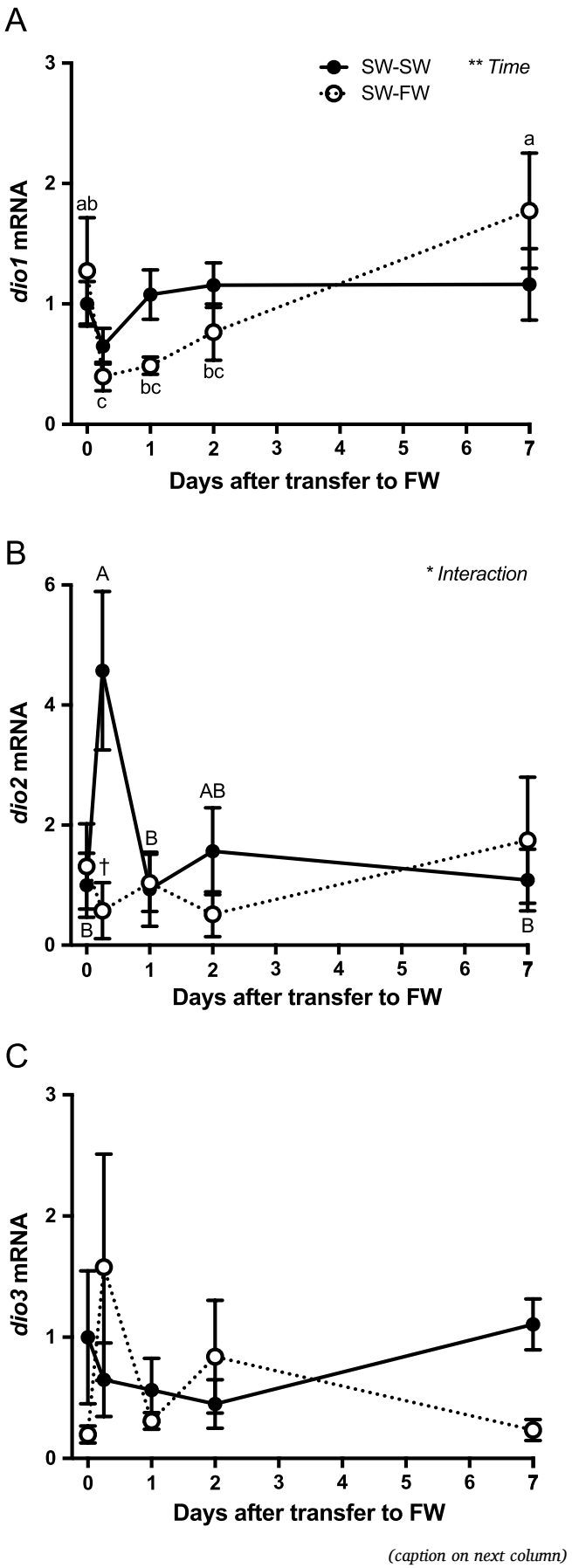
Upon detecting changes in *dio* expression following transfer to FW in vivo, we investigated whether *dio* expression directly responds to changes in extracellular osmolality by incubating gill filaments in isosmotic (330 mOsm/kg), hyposmotic (280 mOsm/kg) or hyperosmotic (380 and 450 mOsm/kg) media for 3 and 6 h. The gene expression of all three *dios* was insensitive to the range of tested extracellular osmotic conditions (Fig. 4A–C).

3.5. Effects of hypophysectomy and TSH replacement on plasma thyroid hormones and branchial deiodinase gene expression

Since *dio* expression did not directly respond to extracellular osmotic conditions, we hypothesized that *dio* transcripts may be primarily regulated by systemic signals. To address whether branchial *dios* are regulated by the pituitary-thyroid axis, we injected hypophysectomized tilapia with bTSH. We measured plasma levels of T3 and T4 to validate the effectiveness of hypophysectomy and bTSH treatment. Plasma T3 was reduced in hypophysectomized tilapia; replacement with bTSH restored plasma T3 to sham-operated levels (Fig. 5A). A similar pattern was observed for plasma T4, with hypophysectomy lowering plasma T4 and treatment with bTSH restoring levels to those of sham-operated fish (Fig. 5B). When compared with sham-operated animals, hypophysectomized animals exhibited reduced *dio1* and *dio2* expression (Fig. 5C and D). *dio3* expression was not affected by hypophysectomy (Fig. 5E). Branchial *dio1* and *dio2* were both stimulated by bTSH injection (Fig. 5C and D). *dio2* returned to levels in the sham-operated group while *dio1* increased 3-fold compared with sham-operated animals. bTSH did not elicit a response on branchial *dio3* expression (Fig. 5D).

4. Discussion

In fishes, the activity of the thyroid axis at the level of its individual components has been largely examined in the context of development, reproduction, and major life-history transitions (Blanton and Specker, 2007; Irachi et al., 2021; Miwa et al., 1988; Specker, 1988). Connections between the thyroid axis and osmoregulatory processes in fishes,



(caption on next column)

Fig. 3. Gene expression of *dio1* (A), *dio2* (B), and *dio3* (C) after transfer of Mozambique tilapia from SW to FW (open circles). Symbols represent mean ± S.E.M (*dio1*, $n = 5–8$; *dio2*, $n = 5–8$; *dio3*, $n = 6–8$; *ef1a*, $n = 6–8$). Control fish were maintained in SW (solid circles) and sampled on the same time course as fish subjected to a FW challenge. Differences among groups were evaluated by two-way ANOVA. Main and interaction effects are marked with *, ** representing $P < 0.05$ and 0.01, respectively. Significant effects between means were followed up by Fisher's protected LSD test. † denotes significant differences from corresponding SW–SW controls at $P < 0.05$, and different letters correspond to significant differences in means across time within SW-FW (lower case) and SW-SW (upper case) groups.

however, are less clear. Most studies that investigated thyroid function within the context of salinity acclimation reported that THs are elevated in response to hyperosmotic conditions (Grau, 1988; Knoeppel et al., 1982; McNabb and Pickford, 1970; Parker and Specker, 1990; Prunet et al., 1989; Specker, 1988; Specker and Kobuke, 1987; Young et al., 1989). Given that THs promote specific processes suited to SW conditions (Refstie, 1982; Saunders et al., 1985; Shrimpton and McCormick, 1999), we reasoned that THs may be attenuated when euryhaline species acclimate to FW conditions. This systemic pattern of regulation, however, may be subject to local modulation; TH metabolism may provide support, through branchial dio activation, to ionocyte remodeling during a transition to FW. We transferred tilapia from SW to FW and observed sharp and sustained declines in plasma T3 and T4, while in the gill dio2-like activity was transiently increased by 6 h after exposure to FW. By contrast, branchial dio1-like activity did not change. To our knowledge, this is the first documentation of systemic and local regulation of TH signaling in a euryhaline fish during the initial stages of FW acclimation.

The rapid decreases in plasma T4 and T3 (Fig. 1) suggest strong hyposmotically-induced suppression of TH production and deiodination. These changes in circulating TH levels may impact multiple physiological systems. For example, methimazole-induced hypothyroidism impaired the growth of Nile tilapia (Van der Geyten et al., 2001). In light of the plasma T3 and T4 patterns reported here, the findings of Van der Geyten et al. (2001) are consistent with the reduced growth rates of Mozambique tilapia reared in FW versus SW (Sparks et al., 2003; Zikos et al., 2014). The range of processes controlled by THs reflect the spatiotemporal regulation of *dio* genes and the enzymatic activities of their encoded proteins. The conversion of circulating T4 into active T3 is attributed to the ORD activities of dio1 and dio2 (Peeters and Visser, 2017). In mammals, hepatic dio1 is associated with the deiodination of T4 to maintain T3 levels in circulation, while dio2 predominantly controls the tissue-specific, localized conversion of T4 into T3 (Marsili et al., 2011). In Atlantic salmon (*Salmo salar*) undergoing parr-smolt transformation (preparatory phase that precedes the transition from FW to SW environments), T4 ORD was elevated in several tissues; hepatic ORD activity was highly correlated with plasma T3 levels and therefore seemingly determined plasma T3 levels (Morin et al., 1993). In mummichogs, hepatic *dio2* mRNA and dio2 activity increased in liver explants in response to a hyposmotic stimulus, an effect mediated, at least in part, by an osmotic response element in the *dio2* promoter (Lopez-Bojorquez et al., 2007). Additional studies in euryhaline teleosts, including salmonids, mummichogs, and various tilapias, revealed distinct patterns of dio activity that varied across tissues and environmental salinities (Mol et al., 1993, 1997, 1998; Orozco et al., 2000, 2002). With respect to their sensitives to THs, however, dio activities across species were generally similar (Mol et al., 1998). It is also possible that upon binding to dio2, T4 enhances ubiquitination and subsequent proteasomal degradation of tilapia dio2, as described for mammalian dio2 in tissues that are highly dependent on its activity (Steinsapir et al., 2000; Zavacki et al., 2009). Such substrate-dependent degradation could explain the subsequent drop in dio2-like activity observed after 6 h of transferring fish from SW to FW. Hence, tissue-specific deiodination activities seemingly play an important role

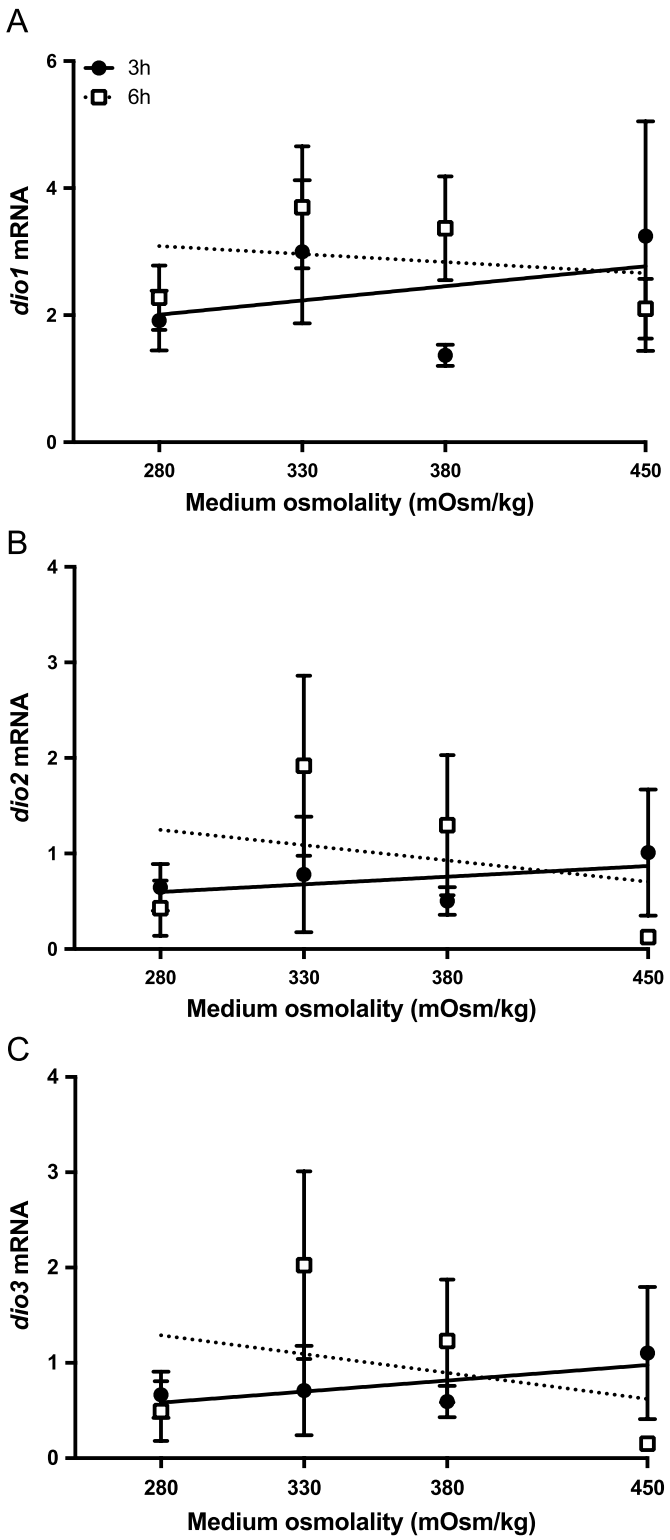


Fig. 4. Gene expression of *dio1* (A), *dio2* (B), and *dio3* (C) in gill filaments exposed to a range of medium osmolalities for 3 h (solid circles) and 6 h (open squares). Symbols represent mean ± S.E.M ($n = 7–8$). Differences among groups were evaluated by linear regression analysis.

in directing environmental responses that require the targeted action of T3.

Since the gill plays a central role in osmoregulation, salinity acclimation entails the remodeling of branchial ionocytes to favor either ion extrusion or absorption when animals are exposed to SW or FW environments, respectively (Kaneko et al., 2008). During acclimation to hyposmotic conditions, the rapid (within 6 h) induction of gene transcripts that encode mediators of ion uptake, such as Na^+/Cl^- cotransporter 2, Clc family Cl^- channel 2c, and Nka alpha 1a, and the suppression of genes encoding effectors of ion extrusion, such as $Na^+/K^+/2Cl^-$ cotransporter 1a and cystic fibrosis transmembrane conductance regulator 1, follows an abrupt drop in environmental salinity (Breves et al., 2017; Inokuchi et al., 2015; Moorman et al., 2015; Tipsmark et al., 2011). We detected a transient increase in total and dio2-like activity (to promote local T3 production) in the gill that temporally overlaped with the timing of branchial remodeling via the differentiation of FW-type ionocytes (Hiroi et al., 2005) (Fig. 2A, C). Inasmuch as THs stimulate cell differentiation in various vertebrate organs (Brown, 1997), it now remains to be determined whether T3 promotes the differentiation of FW-type ionocytes. Ionocytes are strong candidates for regulation by THs in tilapia given that T3 stimulated branchial Nka activity (Peter et al., 2000). THs may also enhance the sensitivity of the gill to other hormones, especially factors known to regulate osmoregulatory activities such as cortisol (McCormick, 2001). For example, T4 potentiated a cortisol-induced increase in branchial Nka activity (Dangé, 1986). The early and transient induction of dio2 activity, therefore, may further contribute to branchial reconfiguration during FW-acclimation through TH's interactions with other hormones.

The regulation of *dio* genes in vertebrates is notably complex and reflects the essential roles they play in a range of biological functions (de Jesus et al., 2001; García-Domingo et al., 1999; Gereben and Salvatore, 2005; Larsen and Zavacki, 2012; Milesi et al., 2017; Williams and Bassett, 2011). Unlike dio activity, the effects of FW acclimation on branchial *dio* gene expression were variable over the experimental time course (Fig. 3). A salinity effect was only observed for *dio1*, where expression was transiently decreased at 6 h followed by a gradual rise to pre-transfer levels by 7 d (Fig. 3A). Interestingly, a transient surge in *dio2* at 6 h was observed in SW-SW controls (Fig. 3B), a response that could have been triggered by other environmental factors, such as time of day. We previously reported that branchial expression of all three *dios* was higher in steady-state FW- versus SW-acclimated tilapia (Seale et al., 2014). Moreover, *dio3*, which did not show a consistent pattern of expression in the current experiment, was transiently upregulated and downregulated following previous FW- and SW-challenge experiments, respectively (Seale et al., 2014). At first, the transient downregulation of *dio1* in FW appears consistent with the observed patterns of plasma TH, where concerted suppression of ORD (via dio1) would result in the lowering of active T3. Nonetheless, while the current pattern of branchial *dio1* expression stands out for matching the time course of changes in total circulating TH and branchial dio enzymatic activity, the nature of the *dio1* and *dio2* responses to a hyposmotic environment were different than those of dio1-and dio2-like activities. To some extent, such discrepancies may be influenced by the use of low and high Km activities to distinguish between the predominant ORD, where some dio1 and dio2 activities may still occur at a low and high Km, respectively. The variability in the expression of *dios* may also be linked to more than one environmental cue. In Atlantic salmon, for example, branchial *dio2a* was induced following transfer to SW and associated with the metabolic response to osmotic stress (Lorgen et al., 2015). By contrast, its paralog, *dio2b*, was induced by increasing day length in both the gill and brain, consistent with the anticipatory nature of TH metabolism that is involved in seasonal life history transitions such as smoltification (Lorgen et al., 2015). Moreover, IRD activity of dio1 or dio3 may also respond to environmental cues that modulate local TH metabolism, though activity levels of dio3 in tilapia were found to be several-fold higher in brain than in gill (Sanders et al., 1999). These differences in regulation illustrate the level of complexity regarding the transcriptional control of *dios* as determined by either an immediate response to environmental salinity or one, cued by other environmental factors such as day light (Wambiji et al., 2011), that prepares the animal for developmental changes.

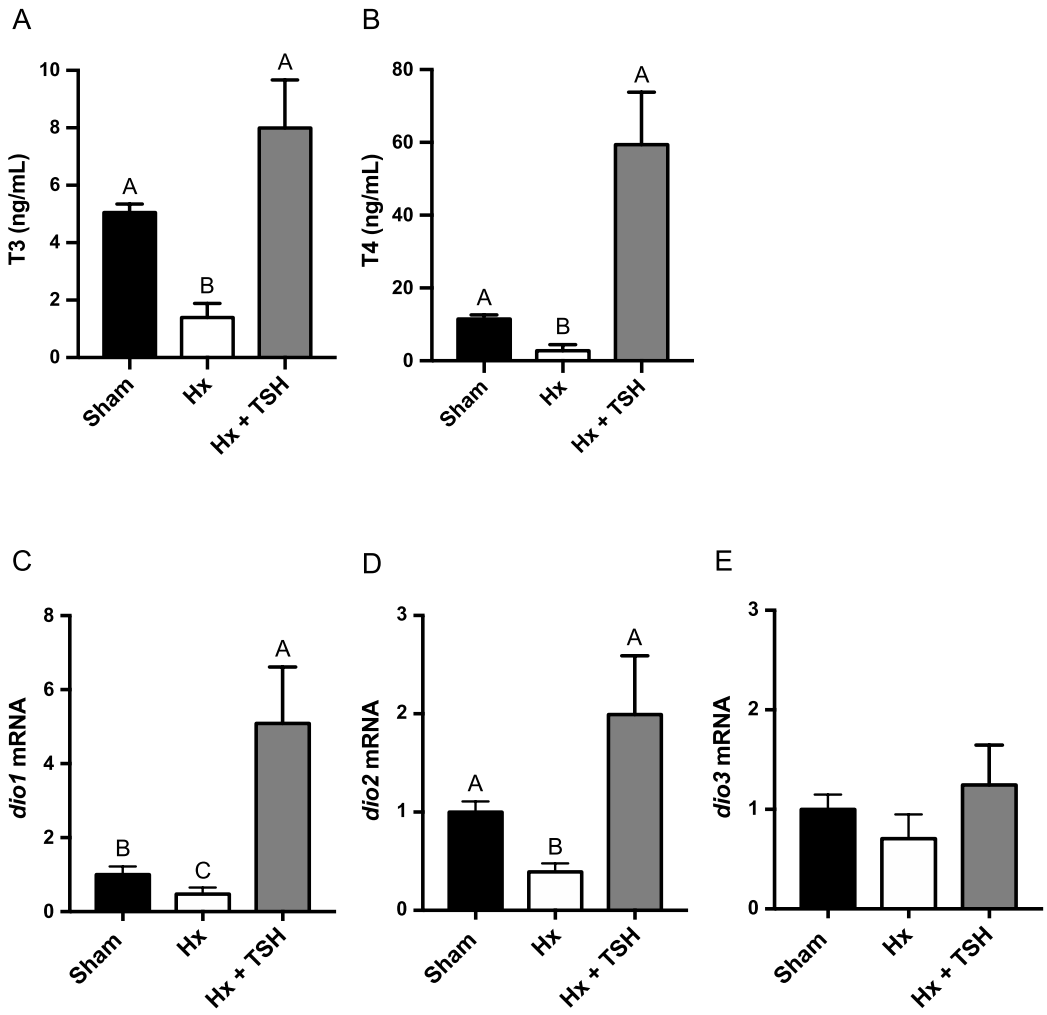


Fig. 5. Plasma levels of T3 (A), T4 (B), and branchial gene expression of *dio1* (C), *dio2* (D), and *dio3* (E) after hypophysectomy of Mozambique tilapia and TSH replacement. Bars represent mean ± S.E.M (n = 4–8). Hypophysectomized fish were sampled 12 h after receiving a single injection of bTSH (shaded bars). Sham-operated (solid bars) and hypophysectomized (open bars) fish receiving saline injections served as controls. Differences among groups were evaluated by one-way ANOVA followed up by Fisher's protected LSD test. Significant differences between means are represented by different letters and correspond to $P < 0.05$.

To discern whether the effects of salinity transfer on the expression of *dios in vivo* occurred in direct response to a change in extracellular osmolality *per se*, or were influenced by systemic cues, we examined the osmotic responsiveness of *dio* genes *in vitro*. Gill filament incubations have previously allowed us to detect the osmotic responsiveness of a series of genes (Inokuchi et al., 2015). In the current experiment, however, the expression of *dios* was unresponsive to physiologically relevant extracellular osmolalities ranging between 280 and 450 mOsm/kg (Fig. 4). It is worth noting that while osmotic response elements motifs were identified in the promoter region of mummichog *dio2* (Lopez-Bojorquez et al., 2007), the osmotic concentration of hyposmotic media employed to induce responses in hepatic mRNA levels and activity in that study was far below the lowest plasma osmotic concentration experienced by Mozambique tilapia facing a direct transfer from SW to FW. These findings suggest that changes in *dio* expression following transfer to FW are regulated by systemic factors.

To address the systemic control of branchial *dio* expression, we targeted the pituitary gland, the major site of TSH synthesis and secretion. Hypophysectomy decreased plasma THs and branchial expression of *dio1* and *dio2*, but not *dio3*. Hormone replacement with TSH restored circulating levels of both T4 and T3 and stimulated *dio1* and *dio2* expression to levels above those of sham-operated fish (Fig. 5A and B), indicating that these two *dios* are regulated by TSH and/or any number of TSH-responsive factors. It is unclear, however, if *O. mossambicus* expresses a receptor for TSH in the gills, and whether signaling through the TSH receptor affects *dio* expression. In mummichog, plasma T4 was reduced by hypophysectomy and shown to increase following injection with ovine TSH (Brown and Stetson, 1983; Grau and Stetson, 1977b). Thus, TSH-induced TH release is a plausible link between the pituitary and branchial *dios*. The observed pituitary-dependent responses in branchial *dio* transcription are also consistent with the reduction of branchial *dio1* that mirrored the concomitant reductions in circulating THs following the transfer of fish from SW to FW. At the systemic level, however, a range of complex interactions may occur between factors within the thyroid axis and other hormones linked with salinity acclimation. For instance, PRL is released from the pituitary of euryhaline fishes in response to reductions in extracellular osmolality that occur following exposure to FW (Seale et al., 2012b), and studies in mummichog showed that injections of ovine PRL prevented the ovine TSH-induced rise in circulating T4 through suppression of thyroid function rather than through changes in peripheral TH activity (Brown and Stetson, 1983; Grau and Stetson, 1977a). Given that PRL rises within 6 h after tilapia are transferred from SW to FW (Seale et al., 2002, 2006), PRL may inhibit the release of T4 from the thyroid and thereby affect plasma T4 levels. The extent to which hormonal interactions may be at play, and whether the branchial expression of *dios* is directly and/or differentially regulated by THs and TSH in Mozambique tilapia, however, remain to be investigated.

In conclusion, our results suggest that TH signaling is locally modulated in the gill during the FW acclimation of Mozambique tilapia. This modulation is achieved through the regulation of branchial deiodinase gene expression and activity. Furthermore, with the concomitant reductions in circulating THs, it is apparent that systemic regulation of TH metabolism is also associated with a hyposmotic challenge. We propose that acute changes in branchial dio activity, and thus tissue T3 levels, support the functional remodeling of the gill during FW acclimation. The next challenge is to identify specific branchial processes that respond to TH signaling to improve the collective understanding of how discrete hormones underlie euryhalinity in fishes.

Declaration of competing interest

The authors declare that there is no conflict of interest that could be perceived as hindering the impartiality of the research reported.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2021.111450.

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