

Thyroid hormone regulation of mRNAs encoding thyrotropin β -subunit, glycoprotein α -subunit, and thyroid hormone receptors α and β in brain, pituitary gland, liver, and gonads of an adult teleost, *Pimephales promelas*

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Abstract

Thyroid hormones (THs) regulate growth, morphological development, and migratory behaviors in teleost fish, yet little is known about the transcriptional dynamics of gene targets for THs in these taxa. Here, we characterized TH regulation of mRNAs encoding thyrotropin subunits and thyroid hormone receptors (TRs) in an adult teleost fish model, the fathead minnow (*Pimephales promelas*). Breeding pairs of adult minnows were fed diets containing 3,5,3'-triiodo-L-thyronine (T_3) or the goitrogen methimazole for 10 days. In males and females, dietary intake of exogenous T_3 elevated circulating total T_3 , while methimazole depressed plasma levels of total thyroxine (T_4). In both sexes, this methimazole-induced reduction in T_4 led to elevated mRNA abundance for thyrotropin β -subunit (*tsh β*) in the pituitary gland. Fish treated with T_3 had elevated

transcript levels for TR isoforms α and β (*tr α* and *tr β*) in the liver and brain, but reduced levels of brain mRNA for the immediate-early gene basic transcription factor-binding protein (*bteb*). In the ovary and testis, exogenous T_3 elevated gene transcripts for *tsh β* , glycoprotein hormone α -subunit (*gph α*), and *tr β* , while not affecting *tr α* levels. Taken together, these results demonstrate negative feedback of T_4 on pituitary *tsh β* , identify *tr α* and *tr β* as T_3 -autoinduced genes in the brain and liver, and provide new evidence that *tsh β* , *gph α* , and *tr β* are THs regulated in the gonad of teleosts. Adult teleost models are increasingly used to evaluate the endocrine-disrupting effects of chemical contaminants, and our results provide a systemic assessment of TH-responsive genes during that life stage.

Introduction

The hypothalamic–pituitary–thyroid (HPT) axis regulates metabolism and growth, reproduction, and brain development in vertebrates. Similarly, in fish, thyroid hormones (THs) have been demonstrated to regulate growth (Huang *et al.* 1996, Power *et al.* 2001, Kang & Chang 2004), influence morphological development (Reddy & Lam 1992, Brown 1997, Tagawa & Aritaki 2005, Lema & Nevitt 2006), and mediate the transition from larval or juvenile forms in a similar manner to how THs regulate metamorphosis in amphibians (de Jesus *et al.* 1998, Trijuno *et al.* 2002, Shiao & Hwang 2006). In addition to their well-established roles in influencing growth and morphology, THs have been shown to promote neural stem cell proliferation in the olfactory system of salmon indicating a role for THs in neurogenesis (Lema & Nevitt 2004), and to mediate the rheotactic shifts

and migratory behaviors of salmon and other diadromous fishes (McCormick *et al.* 1998, Edeline *et al.* 2005). THs also appear to influence reproductive function in teleost fish, since inhibition of endogenous TH production during the time of gonadal germ cell differentiation leads to an increased density of Sertoli and germ cells per cyst in the testis (Matta *et al.* 2002).

While these and other studies have established diverse roles for THs in the growth, development, and behavior of fish (reviewed by Power *et al.* 2001, Yamano 2005), little is known about gene targets for TH action in teleosts. Few TH-responsive genes have been detected in fish (Liu *et al.* 2000, Marchand *et al.* 2004, Manchado *et al.* 2009), yet the identification of these genes is crucial to understanding which molecular pathways mediate TH-regulated responses. Teleost fishes are now commonly used as vertebrate models for assessing the physiological and behavioral impacts of

chemical contaminants, and the adults of several teleost species are used for screening the endocrine-disrupting effects of pollutants (Ankley & Johnson 2004, Ankley & Villeneuve 2006). As teleost models are increasingly employed to test for endocrine disruption of reproduction, brain development, and behavior, it has become clear that a detailed understanding of HPT axis regulation in teleosts is necessary to fully evaluate the thyroid-disrupting impacts of environmental chemicals (Brown *et al.* 2004, Crofton 2008). While several teleost models have been used to examine the effects of contaminants on thyroid status and function (Picard-Aitken *et al.* 2007, Lema *et al.* 2008a), our understanding of HPT regulation in teleosts – and of the molecular mechanisms by which THs regulate specific functions in target tissues – is presently insufficient to use teleost fishes to assess the impacts of chemicals disrupting the thyroid system (Brown *et al.* 2004, Blanton & Specker 2007).

In this study, we examined TH regulation of mRNAs encoding several known or putative TH-responsive genes in the tissues of the adult fathead minnow (*Pimephales promelas*). The fathead minnow is commonly used as an adult teleost model for assessing the toxic and endocrine-disrupting effects of chemical pollutants (USEPA 2002, Ankley & Villeneuve 2006), yet TH-regulated gene targets have not been described in this species. Specifically, we examined TH regulation of mRNAs for thyrotropin β -subunit (*tsh β*) and glycoprotein hormone α -subunit (*gpha*) – which encodes the two protein subunits of the functional thyrotropin (Tsh) hormone – in the pituitary and gonads. We also examined the TH regulation of gene transcripts for the thyroid hormone receptors (TRs) α and β (*tra* and *tr β*) in the brain, liver, and gonads, as well as transcript for basic transcription element-binding protein (*bteb*) in the brain. The *bteb* gene encodes a zinc-fingered transcription factor that binds GC-box domains to regulate TH-mediated gene transcription. Previously, *bteb* has been shown to be TH regulated in the brain of mice (Denver *et al.* 1999) and in the brain, intestine, and tail of *Xenopus* (Furlow & Kanamori 2002, Hoopfer *et al.* 2002), but it has not been tested whether *bteb* is TH regulated in the neural tissues of adult teleosts.

Materials and Methods

Animals and housing

Fathead minnow (*P. promelas*) adults (mean body mass: 2.92 ± 0.15 g; mean fork length: 57.74 ± 0.89 mm) were obtained from Environmental Consulting & Testing (Superior, WI, USA) and held in 185 l, flow-through (0.5 l/min) aquaria prior to commencing the experiment. Minnows were maintained under a 16:8 L/D photoperiod with water quality parameters of 24–26 °C, 6.6–7.4 mg/l dissolved oxygen, and 8.1–8.3 pH for the duration of the

experiment. All animals were maintained in accordance with established guidelines of the Institutional Animal Care and Use Committee of Battelle.

Dietary methimazole and T₃ treatments

Methimazole and 3,5,3'-triiodo-L-thyronine (T₃; Sigma) were administered to breeding pairs of adult minnows using a commercial fish pellet diet (BioDiet Grower pellets, BioOregon, Warrenton, OR, USA). Methimazole (125 mg) and T₃ (5 mg) were dissolved in alkaline ethanol solution (33 ml 95% ethanol and 12 ml 0.1% NaOH), and sprayed on the pellet feed (50 g) using a Badger 350 air brush (Badger Air-Brush Co., Franklin Park, IL, USA) with nitrogen as a vehicle. Pellet feed for the control treatment was sprayed with alkaline ethanol solution only.

Adult fathead minnows were placed in 38 l aquaria, with one adult male and one adult female per aquarium ($n=8-16$ pairs per treatment group). Each aquarium also contained a 10.2 cm diameter clay pot that was split longitudinally to provide spawning substrate for the pair. Minnow pairs were fed methimazole, T₃, or control pellet feed (0.3 g/pair per day) once per day between 0900 and 1100 h for 10 days, to generate approximate doses of methimazole (0.75 mg/pair per day) and T₃ (0.03 mg/pair per day) comparable with those used previously with coho salmon (*Oncorhynchus kisutch*; Larsen *et al.* 1997). Each pair was also given two additional feedings of frozen *Artemia* brine shrimp twice per day, between the hours of 1400–1500 and 1700–1800. Spawning activity during the 10-day period was monitored each morning ~ 3 h after the start of the light photoperiod by checking the spawning clay pot substrate and counting the number of eggs present.

After the 10 days of dietary exposure to methimazole or T₃, minnows were euthanized (tricaine methanesulfonate, MS222; Argent Chemical, Redmond, WA, USA), and body mass (g) and fork length (mm) measured. Plasma was collected, and the pituitary gland, brain, liver, and gonads were dissected. The gonads were weighed separately to determine gonadosomatic index (GSI) as calculated by the following formula: (tissue mass/(body mass – tissue mass)) $\times 100$. The pituitary, brain, liver, and one gonad were frozen rapidly in liquid nitrogen. All tissues were stored at -80 °C. The other gonad was immersed in Bouin's fixative for histological staging.

T₄ and T₃ RIAs

Plasma concentrations of thyroxine (T₄) and T₃ were measured by RIA as described previously (Dickhoff *et al.* 1982). Anti-L-T₄ serum (1:4000) and anti-L-T₃ serum (1:10 000) were obtained from Accurate Chemical & Scientific Corp. (Westbury, NY, USA). The intra-assay coefficient of variation was 4.52% for the T₄ assay and 4.77% for the T₃ assay. Serial dilutions of plasma were parallel to the standard curve for both assays.

Reverse transcription

Total RNA was extracted from the pituitary using the MiniPrep RNeasy Kit (Qiagen, Inc.), and from the brain, liver, and gonads using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). Extracted RNA was quantified by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA), and diluted to 15 ng/ μ l. Total RNA was reverse transcribed (RT) in 15 μ l reactions containing 3.0 μ l 5 \times buffer and 1.5 μ l of 0.1 M dithiothreitol (Invitrogen), 0.75 μ l dNTP (stock of 10 mM each of dCTP, dGTP, dTTP, and dATP) and 0.255 μ l random hexamer (500 ng/ μ l stock, Promega), 0.3 μ l RNase inhibitor (20 U/ μ l, Applied Biosystems, Inc., Foster City, CA, USA), 0.1875 μ l Superscript II Reverse Transcriptase (200 U/ μ l, Invitrogen), 6.0375 μ l ddH₂O (nuclease-free water, Sigma), and 3.0 μ l of total RNA template (15 ng/ μ l). All RT reactions were performed in 96-well plates on a thermal cycler (iCycler, Bio-Rad Laboratories Inc.) under a thermal profile of 25 °C for 10 min, 48 °C for 60 min, and 95 °C for 5 min.

Real-time quantitative RT-PCR assays

Primers and Taqman probes for real-time quantitative RT-PCR assays were designed to *tsh β* (Genbank accession no. DQ677879; Lema *et al.* 2008b), *gpha* (Genbank accession no. DQ256072; Villeneuve *et al.* 2007), *tra* (Genbank accession no. DQ074645) and *tr β* (Genbank accession no. AY533142; Filby & Tyler 2007), and *bteb* (Genbank accession no. EF432310; Lema *et al.* 2008a) from fathead minnow (Table 1). Primers and probes for all quantitative RT-PCRs were designed using Primer Express software (ABI) and synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Quantitative RT-PCRs were run in 25 μ l volumes with each reaction containing 12.5 μ l Master Mix (ABI Universal MasterMix Reagent), 0.5 μ l forward primer (45 μ M), 0.5 μ l reverse primer (45 μ M), 0.5 μ l probe (10 μ M), 8.0 μ l ddH₂O (nuclease-free water, Sigma), and 3.0 μ l of RT cDNA template. Reactions were run on an ABI 7700 Sequence Detector under a PCR thermal profile of 50 °C for 2 min, 95 °C for 10 min, and then 40–45 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples for each gene were run on a single 96-well plate. For each gene measured, we tested for DNA contamination by analyzing total RNA samples that were not RT; no amplification was observed in any of these samples over 45 cycles. Serial dilution of a single total RNA from the experiment was used as a standard curve reference, and correlation coefficients of the standard curves ranged from 0.98 to 1.00. PCR efficiencies for each gene were calculated from the standard curves using the following formula: $E = 10^{(-1/\text{slope})}$ (Rasmussen 2000) and are presented in Table 1. All standard curve samples were run in triplicate, while experimental samples themselves were not run in duplicate. Each run included duplicate samples lacking cDNA template to further check for DNA contamination during RNA preparation. As an internal reaction control for each gene of interest, we quantified transcript levels of *18s* (QuantumRNA Universal 18s, ABI). Within each tissue, transcript levels of *18s* were similar across all treatments and sexes. Transcript levels for genes of interest were calculated using the serially diluted standard curve and were expressed relative to *18s* mRNA levels in the given sample. The relative level of gene transcript was then calculated by dividing the above values by the mean of the male control group (Pfaffl 2001). This calculation provides a clear representation of the relative changes in expression of each gene among treatments and sexes.

Table 1 Nucleotide sequences for primers and Taqman probes used in quantitative real-time reverse transcription-PCR

	Primer or probe	Sequence	Amplicon size (bp)	PCR efficiency (average)
Transcript				
	<i>tshβ</i>	Forward primer (5'–3')	GGTGCAGCCTCTCTGAACCA	73
	Probe (5'–3')	AACGAGGACCCACCAACTCCTTCACA		
<i>gpha</i>	Reverse primer (5'–3')	CTTCTGCTTCTCCAGGGACAGT	72	2.0373
	Forward primer (5'–3')	CACCCCTGAGGTCCAAGAAA		
<i>tra</i>	Probe (5'–3')	CCATGCTCGTTCCTCAAAATATCACATCA	83	2.0360
	Reverse primer (5'–3')	TGGCAACACAGCATGTAGCTT		
<i>trβ</i>	Forward primer (5'–3')	TGCAGGCTGTACTCCTCATGA	74	2.0477
	Probe (5'–3')	AGATCGTTCTGGACTGACATGTGTGGAAAAGAT		
<i>bteb</i>	Reverse primer (5'–3')	CAGGTACGTCTCCTGACACTTCTC	70	1.9880
	Forward primer (5'–3')	TTGCTCCAAGCCGTGATTCT		
	Probe (5'–3')	CTTTCCTCTGATCGTCCAGGTTTAAACGAGC		
	Reverse primer (5'–3')	TGACAACGCTCTATCCGCTCTA		
	Forward primer (5'–3')	CAAACCGGCGTAAAGGAAAA		
	Probe (5'–3')	CGGGAGAAATGCAGGGTAAAAGGAC		
	Reverse primer (5'–3')	CATGCAGTCTGTACAGTTCCA		

Gonad histology

Gonad samples were immersed in Bouin's fixative (24 h), transferred to 70% ethanol, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned longitudinally (5 μ m). Gonad stages were quantified from three tissue sections from each gonad; the first section was collected approximately halfway through the tissue with two additional sections collected at subsequent 200 μ m intervals. Sections were stained with hematoxylin and eosin, and gonadal stages of spermatogenesis and oogenesis were quantified by stereology as described previously (Leino *et al.* 2005).

Statistical analyses

Two-factor ANOVA models with treatment, sex, and treatment \times sex interaction as factors were used to examine whether methimazole or exogenous T₃ affected plasma total T₄ and T₃. For each tissue, we used two-factor ANOVAs with treatment and sex as factors to determine whether mRNA levels of *tsh β* and *gph α* in the pituitary gland, and of *tr α* and *tr β* in the brain, liver, and gonad, varied by treatment and/or sex. When a significant effect of treatment was found, multiple pairwise comparisons were made between the control and each treatment using Dunnett's tests. Transcript and hormone values that failed to conform to the assumptions of normality were square root transformed prior to analysis. Sample values that exceeded three S.D.s from the mean were considered outliers and excluded.

One-factor ANOVA models were used to test for differences in spawning frequency and the mean number of eggs produced. We then used χ^2 tests to compare the mean distribution of stages of spermatogenesis or oogenesis between treatments. Exact *P* values for the χ^2 tests were obtained using StaTable 1.0.1 (Cytel Software Corp., Cambridge, MA, USA). To identify which stages were generating treatment differences, we conducted multiple pairwise comparisons (*t*-tests) between the control and each treatment within each stage class. Critical probability levels (α) for these *t*-tests were Bonferroni corrected for the total number of pairwise comparisons within a sex. The relationship between gonad staging and the time since last spawning was examined using ANCOVA models, as was the relationship between gonad staging and relative transcript abundance.

Results

Plasma THs

Plasma total T₄ levels were reduced by methimazole in both sexes (Fig. 1A; treatment, $P < 0.0001$; treatment \times sex interaction, $P = 0.039$). T₄ levels also differed between the sexes, with males having higher levels than females ($P = 0.029$). Dietary T₃ intake elevated plasma total T₃ in

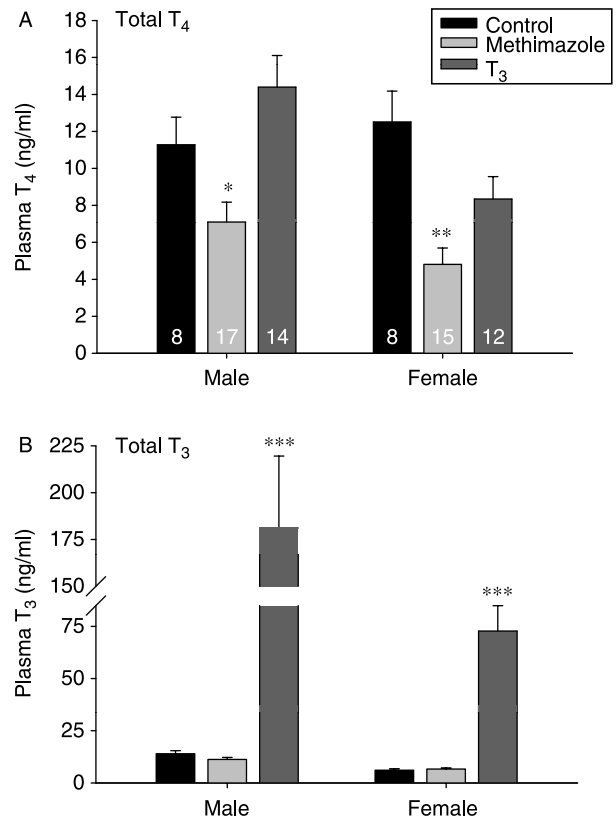


Figure 1 Dietary intake of methimazole and T₃-altered peripheral levels of total T₄ (A) and total T₃ (B). Methimazole depressed total T₄ in males and females, while exogenous T₃ significantly elevated total T₃ in plasma in both sexes. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$, compared with control of same sex.

males and females (Fig. 1B; treatment, $P < 0.0001$; treatment \times sex interaction, $P < 0.0001$), although males had greater levels of total T₃ than females across all treatments ($P < 0.0001$).

TH regulates pituitary genes encoding thyrotropin

Gene transcripts for *tsh β* in the pituitary gland were elevated in males and females treated with methimazole (Fig. 2A; $P = 0.0003$). Males also had greater levels of pituitary transcript for *tsh β* than females ($P = 0.042$). Transcript levels of *gph α* varied among treatments (Fig. 2B; $P = 0.049$), with a slight elevation in methimazole-treated fish. Levels of *gph α* mRNA were higher in males than females ($P = 0.045$).

T₃ upregulates *tr α* and *tr β* mRNAs in brain

In the brain, transcripts for *tr α* ($P < 0.0001$) and *tr β* ($P = 0.0002$) were elevated by exogenous T₃ (Fig. 3A and B); however, the effect on levels of *tr α* mRNA was more pronounced than that of *tr β* . Transcripts for *tr α* increased by

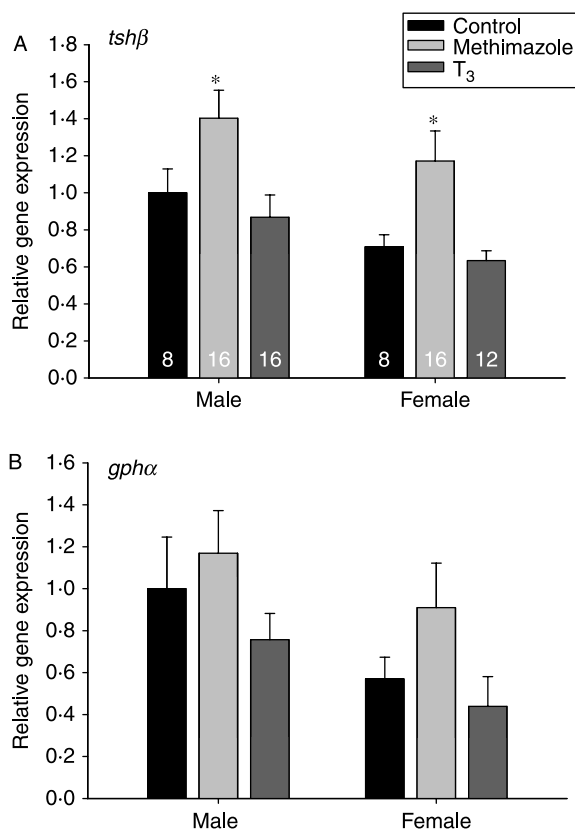


Figure 2 Oral exposure to methimazole elevated relative mRNA levels for thyrotropin β -subunit (*tsh\beta*) (A) and glycoprotein hormone α -subunit (*gph\alpha*) (B) in the pituitary gland. This methimazole-induced increase in *gph\alpha* mRNA, however, was too minor to be significant in pairwise statistical comparison with the control treatment with that sex. * $P < 0.05$, compared with control of same sex.

104%, and for *tr\beta* by 26%, in the brain of T₃-treated males. In females, T₃ induced a 95% increase in *tr\alpha* and 23% elevation in *tr\beta* mRNAs. Neither *tr\alpha* nor *tr\beta* were affected by treatment with methimazole. Transcript levels for *tr\alpha* did, however, differ between sexes with higher levels in the brain of females than in males ($P = 0.05$). Levels of *tr\beta* transcripts were similar between the sexes.

Transcript abundance for *bteb* was reduced 64% in the brain of male minnows by dietary T₃ ($P < 0.0001$), but was unaffected in females (Fig. 3C). This sex difference in the response of brain *bteb* gene expression was reflected as a marginally significant difference in *bteb* transcript between the sexes ($P = 0.069$).

T₃ autoinduces mRNAs for TRs in the liver

Minnows given exogenous T₃ had elevated transcripts for *tr\alpha* in the liver relative to control fish (Fig. 4A; $P < 0.0001$). Transcript abundance for *tr\alpha* was similar in the liver of males and females. By contrast, male minnows had higher levels of

tr\beta transcript in the liver than females ($P < 0.0001$), and although exogenous T₃ induced elevated mRNA levels for *tr\beta* in the liver (Fig. 4B; $P < 0.0001$), the magnitude of the increase varied between sexes ($P = 0.005$). Methimazole did not affect liver *tr\alpha* or *tr\beta* transcripts in either sex.

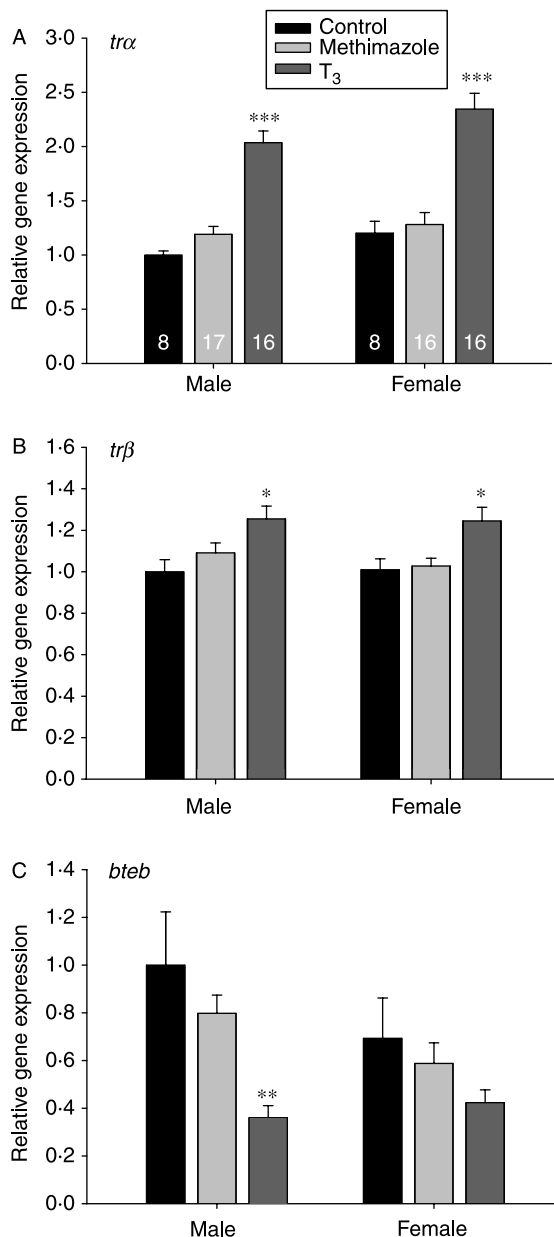


Figure 3 Oral exposure to exogenous T₃ significantly altered gene transcripts for thyroid hormone receptor α (*tr\alpha*) (A) and *tr\beta* (B) in the brain. The 10-day T₃ exposure also resulted in a decline of brain mRNA abundance for basic transcription element-binding protein (*bteb*) (C). This effect of T₃ was pronounced in males, but was not statistically significant in a pairwise comparison with control females ($P = 0.08$). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$, compared with control of same sex.

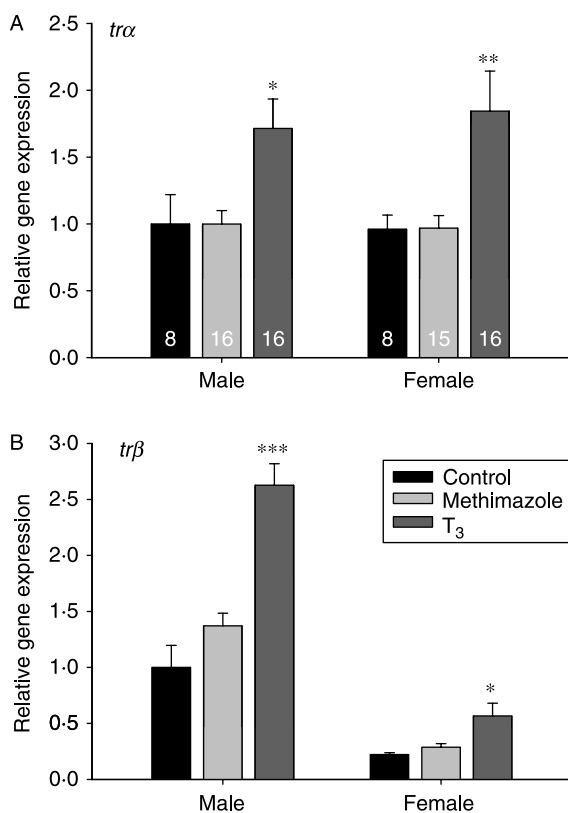


Figure 4 Exogenous T₃ increased relative gene transcripts for thyroid hormone receptor α (*trα*) (A) and for *trβ* (B) in the liver. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$, compared with control of same sex.

Gonad transcript abundance, staging, and reproductive behavior

GSI was greater in males than females ($P < 0.0001$), but was not affected by either methimazole or T₃. There was no difference among treatments in the frequency of spawning or in the mean number of eggs produced per spawning event. Males exposed to either methimazole or T₃ had higher number of cysts containing spermatozoa in their

testis compared to control males (Table 2; methimazole versus control: $\chi^2 = 19.57$, $P = 0.0006$; T₃ versus control: $\chi^2 = 21.04$, $P = 0.0003$). Males from the methimazole and T₃ treatments, however, had similar profiles of spermatogenesis stages in their testes. Females in the methimazole or exogenous T₃ treatments had a reduced frequency of cortical alveolus stage oocytes relative to control females (methimazole versus control: $\chi^2 = 12.02$, $P = 0.007$; T₃ versus control: $\chi^2 = 9.68$, $P = 0.022$). There was no difference in the distribution of oocytes in various stages of oogenesis between methimazole and T₃-treated females.

In the testis, the proportion of cysts containing germ cells at various stages of spermatogenesis was related to the time since last spawning. The number of spermatogonia decreased ($r^2 = 0.38$, $P = 0.041$), and cysts containing spermatozoa increased ($r^2 = 0.52$, $P = 0.019$) with increased days since last spawning. This increase in spermatozoa since last spawning, however, was observed only in control and T₃-treated males; males treated with methimazole did not show this same temporal relationship, suggesting that hypothyroidism may inhibit spermatogenesis in adult fish.

There were higher levels of *tshβ* transcript in testes than ovaries (Fig. 5A; $P < 0.0001$). In response to exogenous T₃, transcripts for *tshβ* were elevated 23% in the testis ($P = 0.002$), but were unaffected in the ovary. Similarly, *gpha* mRNA levels varied between sexes and were elevated 120% in the testis and 112% in the ovary by T₃ (Fig. 5B; treatment, $P < 0.0001$; sex, $P < 0.0001$). Neither methimazole nor T₃ had any effect on mRNA levels for *trα* in either the testis or ovary; *trα* did, however, show a strong sex difference with transcript levels approximately five times greater in testis than in ovary (Fig. 5C; $P < 0.0001$). By contrast, *trβ* transcript was induced by T₃ in the gonadal tissues of both sexes (Fig. 5D; $P < 0.0001$). In the testis, *trβ* transcripts were elevated 204% in T₃-exposed fish, while in the ovary *trβ* transcripts were increased 80%. Overall, the levels of *trβ* transcript were higher in the ovary compared with the testis ($P < 0.0001$).

Examination of the relationship between relative transcript levels in gonadal tissue and gonadal staging revealed that *trβ* mRNAs declined as the proportion of 1° and 2°

Table 2 Mean percentage observed for cyst stages of spermatogenesis and oogenesis in the testis and ovary

	Spermatogonia	1° Spermatocytes	2° Spermatocytes	Spermatids	Mature spermatozoa
Male					
Control	13.6 ± 1.9	24.3 ± 4.1	31.5 ± 5.2	18.7 ± 2.9	12.5 ± 1.3
Methimazole	12.5 ± 2.4	19.0 ± 2.3	26.7 ± 3.3	15.7 ± 1.9	27.2 ± 5.5*
T ₃	13.7 ± 1.7	20.0 ± 2.2	20.4 ± 2.9	19.1 ± 1.8	26.8 ± 5.3*
	Oogonia	Cortical alveola	Early vitellogenic	Late vitellogenic	
Female					
Control	29.2 ± 5.9	38.3 ± 6.4	17.7 ± 3.0	14.7 ± 4.7	
Methimazole	42.8 ± 4.0	23.7 ± 3.9*	19.1 ± 2.5	14.3 ± 2.5	
T ₃	37.4 ± 4.2	25.2 ± 2.9*	16.3 ± 1.8	21.1 ± 4.4	

* $P < 0.05$ compared with control group.

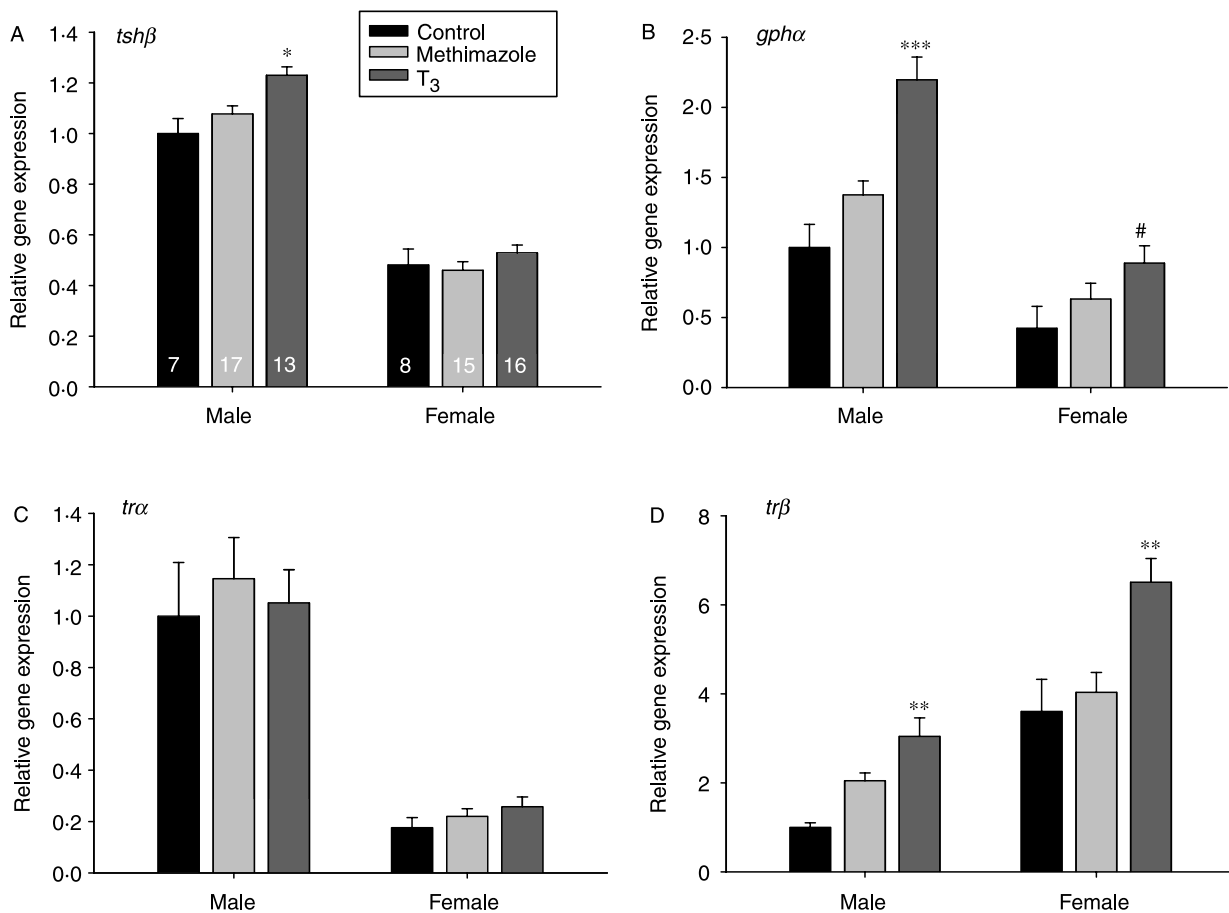


Figure 5 Exogenous T₃ elevated transcript abundance for *tshβ* in the testis (A), *gphα* in the testis and ovary (B), and *trβ* in the testis and ovary (D). Transcript levels for *traα* were unaffected by either methimazole or T₃ treatment (C). # $P=0.05$, * $P<0.05$; ** $P<0.005$; *** $P<0.0001$, compared with control of same sex.

spermatocytes decreased (Fig. 6A), and increased as the proportion of mature spermatozoa in the testis increased (Fig. 6B). The relationships between *trβ* transcript and gonadal staging were similar in control and methimazole-treated males. In the testis of T₃-treated males, however, *trβ* transcript abundance showed a stronger decline relative to the proportion of 1° and 2° spermatocytes ($P=0.036$) and an augmented increase relative to the proportion of mature spermatozoa ($P=0.029$). This result suggests that T₃ not only promotes a shift towards more spermatozoa in the testis (Table 2), but also influences *trβ* transcript abundance independent of T₃-induced changes in spermatogenesis staging.

Discussion

THs regulate a variety of cell functions by facilitating or inhibiting gene transcription, yet little is known about gene regulation by THs in adult teleosts. Here, we examined TH regulation of gene transcripts encoding thyrotropin subunits

in the pituitary and gonads, *traα* and *trβ* in several target tissues, and the transcription factor *bteb* in the brain using the fathead minnow teleost model. Adult male and female minnows exposed to dietary T₃ for a period of 10 days had elevated plasma total T₃ levels, whereas dietary methimazole depressed total T₄. The fathead minnow has been shown to have sexual dimorphism in peripheral levels of THs (Crane *et al.* 2004). Accordingly, we observed higher T₄ and T₃ levels in males than females, although this difference may have been influenced by sex differences in consumption rates of the methimazole and T₃-treated food – which was not controlled within a breeding pair. In both sexes, methimazole and T₃ treatments did, however, alter peripheral T₃ and T₄, which then led to changes in mRNA levels for several TH-regulated genes. In the pituitary gland, transcripts for *tshβ* were elevated in methimazole-treated fish. Male and female minnows also showed changes in mRNAs for *traα* and *trβ* in the brain and peripheral tissues. Exogenous T₃ enhanced transcript abundance for *traα* and *trβ* in the brain of both sexes, while reducing transcript for *bteb* in males only. T₃ also elevated

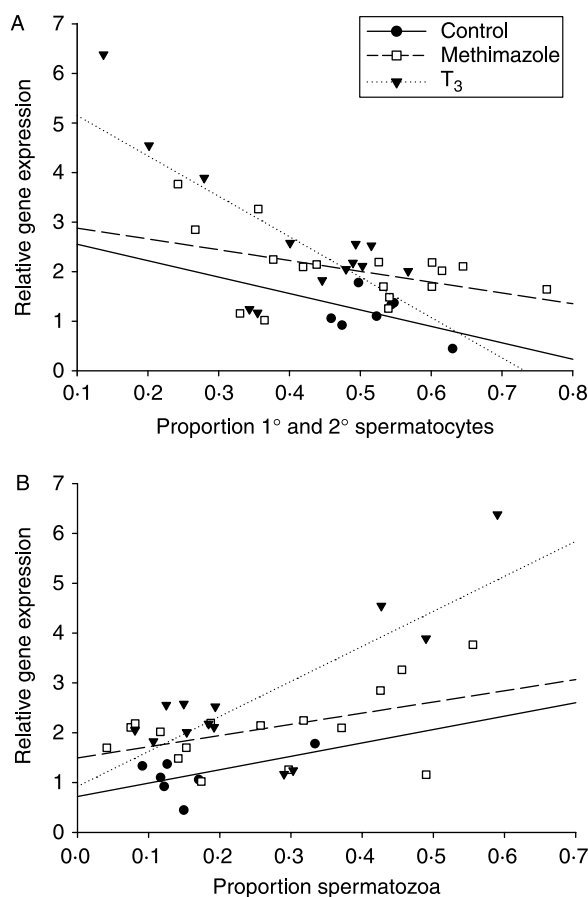


Figure 6 Relative gene transcript levels for thyroid hormone receptor β (*trβ*) in the testis declined relative to the proportion of 1° and 2° spermatocytes (combined) in the testis (A), and increased with a greater proportion of mature spermatozoa (B).

transcripts for *trα* and *trβ* in liver of both sexes, and *trβ* and *gphα* in testis and ovary.

These results demonstrate that transcripts for *tshβ* and *gphα* in the pituitary are upregulated in response to reductions in peripheral T_4 . The transcriptional dynamics of thyrotropin in the pituitary have been examined in several other teleosts (Larsen *et al.* 1997, Pradet-Balade *et al.* 1999, Chatterjee *et al.* 2001), and our results correspond with the findings of those previous studies. For instance, in adult goldfish (*Carassius auratus*) pituitary *tshβ* transcripts were reduced 30% by exogenous T_4 treatment, and elevated to twice control levels in fish exposed to the goitrogen thiourea (Yoshiura *et al.* 1999). Similarly, thiourea depressed circulating T_4 and T_3 and elevated mRNA abundance for *tshβ* and *gphα* in the pituitary of adult European eel, *Anguilla anguilla* (Pradet-Balade *et al.* 1997). Taken together, these responses of pituitary thyrotropin transcripts in teleosts indicate the presence of a negative feedback loop, whereby peripheral TH levels regulate thyrotropin production and, ultimately, TH biosynthesis by

the thyroid gland. Additional support for this negative feedback comes from studies by Leiner & MacKenzie (2003), who found that red drum (*Sciaenops ocellatus*) fingerlings, given aqueous T_3 exposure (100 ng/ml), had reduced peak levels of plasma total T_4 over the diel cycle.

The experimentally induced changes in peripheral THs also altered levels of transcripts for *trα* and *trβ* in the brain and liver – and *trβ* in the gonads – of adult minnows. TRs act as ligand-activated transcription factors by inducing or repressing the transcription of genes containing thyroid response element (TRE)-binding domains in their promoter region (Ribeiro *et al.* 1998). In amphibians, TR genes themselves contain TREs so that transcription of these genes is autoinduced by T_3 (Machuca *et al.* 1995). In fish, TRs are encoded by at least two genes (Marchand *et al.* 2001), and two receptor cDNAs (*trα* and *trβ*) have been identified in the fathead minnow (Filby & Tyler 2007). The receptor isoforms are expressed in sex, tissue, and developmental-stage-specific patterns in these minnows and in other teleosts (Yamano & Miwa 1998, Nelson & Habibi 2006, Filby & Tyler 2007). In zebrafish (*Danio rerio*), for instance, *trα* mRNAs are expressed more highly than *trβ* transcripts during early embryonic development (Liu *et al.* 2000). Yet in adults of this species, *trβ* mRNA levels are greater in the brain, while *trα* mRNA is more abundant in liver and ovary.

Here, transcripts for *trα* and *trβ* were elevated by T_3 in the brain and liver of both sexes of fathead minnow. To our knowledge, this is the first demonstration that transcripts for TRs are regulated *in vivo* by T_3 in the brain and liver of adult teleosts. In the context of previous studies in teleosts, the autoinduction of *trα* and *trβ* mRNAs by T_3 appears consistent across developmental stages after embryogenesis. In zebrafish embryos, *trα* mRNAs appear to be T_3 regulated while *trβ* is not (Walpita *et al.* 2007), but in larvae of this species transcripts for *trα1* and *trβ1* have both been shown to be upregulated *in vivo* in a dose-dependent manner by T_3 (Liu *et al.* 2000). In Senegalese sole (*Solea senegalensis*), mRNAs for *trβ* are elevated during larval metamorphosis, and experimental manipulations revealed that *trβ* transcript levels increase in response to exogenous T_4 – and decrease in response to thiouracil – during the metamorphic transition in this flatfish (Manchado *et al.* 2009). Transcripts for conger eel (*Conger myriaster*) *trαA* and *trαB* expressed *in vitro* in eel (*A. anguilla*) hepatocytes were induced equally by T_3 and T_4 , while transcripts for *trβA* and *trβB* were both upregulated more strongly by T_3 than T_4 (Kawakami *et al.* 2006). In other vertebrates, TR mRNAs are clearly regulated by THs, but whether these genes are induced or repressed depends on the tissue, receptor isoform, and taxon (Hodin *et al.* 1990, Kanamori & Brown 1992, Machuca *et al.* 1995, Sadow *et al.* 2003). In *Xenopus*, *trβ* is autoinduced by T_3 in the brain (Krain & Denver 2004), while in mice, T_3 inhibits gene transcription for *trβ1* and *trα2* in the liver, but induces transcription of these genes in the heart (Sadow *et al.* 2003). To complement these findings from other vertebrate models,

our results suggest transcripts for *trα* and *trβ* are similarly T₃ induced in the brain and liver of adult teleosts.

In the brain, we also found that the 10-day T₃ exposure downregulated mRNA abundance for *bteb*. Female minnows showed a similar trend towards reduced *bteb* transcript abundance in response to T₃, although this effect was not statistically significant ($P=0.08$). The *bteb* immediate-early gene encodes a zinc-fingered transcription factor that binds GC-box domains to facilitate or inhibit TH-mediated gene transcription. In mammals, the T₃-induced Bteb protein binds the promoter of the *trβ* gene to regulate *trβ* autoexpression by THs (Bagamasbad *et al.* 2008). Bteb has been shown to mediate T₃-induced neural differentiation and neurite branching, and these effects on neurogenesis occur via TH activation of *trβ* (Denver *et al.* 1999, Cayrou *et al.* 2002). In *Xenopus*, *bteb* mRNA has been shown to be upregulated by T₃ in brain and other tissues during metamorphosis (Furlow & Kanamori 2002, Hoopfer *et al.* 2002), while in mammals, T₃ upregulation of *bteb* appears specific to neurons and is only seen during early developmental stages (Denver *et al.* 1999). In both amphibian and mammalian tissues, *bteb* appears to act as an immediate-early gene with T₃ upregulation of *bteb* transcripts occurring rapidly (within 24–72 h; Hoopfer *et al.* 2002). By contrast, we observed a decline in *bteb* transcript abundance in the brain of male and female minnows after 10 days of T₃ treatment. Given the length of T₃ exposure used here, this reduced *bteb* abundance suggests there could be a refractory response of *bteb* to the protracted supraphysiological elevation of peripheral T₃. Nevertheless, this observation suggests that *bteb* may be TH regulated in the brain of teleosts, although additional experiments are needed to test whether T₃ upregulates *bteb* transcription over a shorter duration of T₃ exposure. Moreover, the question of whether *bteb* is TH regulated in neural tissues during early teleost development as it is in mammals and amphibians remains to be addressed.

In the gonads, our results provide the first evidence for T₃ regulation of TR transcripts in an adult teleost. These data also suggest, however, that TRs may have roles in the gonad, which are distinct from their functions in brain and liver. In both the testis and ovary, we found that *trβ* mRNA was elevated by T₃ treatment, although *trβ* abundance was greater in ovaries than testes across all exposure groups. This is consistent with previous studies of sexually mature, adult teleosts where ovaries had higher levels of *trβ* mRNA than testes (Nelson & Habibi 2006, Filby & Tyler 2007). By contrast, *trα* mRNA was expressed at greater levels in testis than ovary, but was unaffected by T₃ in either tissue. The differential regulation of *trα* and *trβ* in the gonads likely indicates distinct roles for these two receptors in the regulation of gametogenesis and gonadal function. Little is known about the functions of TRs in the gonads, however, and why *trα* mRNA was not regulated by THs in these tissues is not clear.

Associations between thyroid status and reproductive function have been known for some time in teleosts (Cyr & Eales 1996), and there is now accruing evidence that THs influence testicular development and spermatogenesis in fish and other vertebrates (Maran 2003, Krassas & Pontikides 2004). In mammals, THs have been shown to regulate Sertoli cell proliferation leading to changes in testis development and, subsequently, sperm production after sexual maturity (Cooke *et al.* 1991, Francavilla *et al.* 1991, Maran & Arudhas 2002). The effects of THs on the mammalian testis appear mediated in part via TR-regulated pathways, since knockout of *trα1* leads to elevated Sertoli cell number, increased testis mass, and heightened daily sperm production (Holsberger *et al.* 2005). These influences of THs on testicular function may not be limited to direct effects on the gonad, as neonatal hypothyroidism also alters the development of GnRH neural pathways leading to changes in pituitary gonadotropin secretion (Jansen *et al.* 2007). Although less well understood, comparable effects of hypothyroidism on testicular function appear to occur in teleosts (Swapna & Senthilkumaran 2007). In the catfish *Clarias gariepinus*, exogenous T₄ reduced plasma and testis testosterone levels and led to fewer spermatids and spermatozoa (Jacob *et al.* 2005), while 21-day thiourea exposures to pre-spawning males led to narrowed seminiferous tubules and fewer spermatozoa (Swapna *et al.* 2006). Hypothyroidism during early life has also been shown in teleosts to result in larger mass testis, more Sertoli cells, and increased spermatozoa after maturation (Matta *et al.* 2002).

There is limited evidence that these effects of THs on testicular function may not occur solely through TR-mediated pathways. Here, we found that T₃ induced an elevation in *tshβ* transcript in the testis, indicating this gene is TH regulated in this tissue. We also observed that variation in testis *tshβ* transcript abundance was associated with progression of spermatogenesis, with higher *tshβ* mRNA levels in testes with a greater proportion of mature spermatozoa. Receptors for Tsh have recently been identified in the testicular tissues of several species of teleost fishes, and transcripts for the receptor appear to be localized to the gametes in at least one of these species (Kumar *et al.* 2000, Goto-Kazeto *et al.* 2003, Rahman *et al.* 2003, Vischer & Bogerd 2003, Rocha *et al.* 2007). Transcripts for *tshβ* have also been shown by RT-PCR and *in situ* hybridization to be present in the testis of another adult teleost: the red-spotted grouper, *Epinephelus akaara* (Wang *et al.* 2004). This species experiences environmentally induced sex change, and Wang *et al.* (2004) observed increases in *tshβ* transcript abundance when the gonadal tissue shifted from ovarian to testicular cell types as individuals changed sex from female to male. Transcripts for *tshβ* in these male grouper were localized to spermatogonia and spermatocytes. While the role of Tsh in the gonads clearly requires further study, our observation that testicular – but not ovarian – *tshβ* mRNA is upregulated by T₃ suggests Tshβ protein may be important to the TH-mediated effects on testicular function and spermatogenesis.

In summary, these results provide the first systematic assessment of TH gene regulation in an adult teleost – the fathead minnow – a species that is commonly used as a model for assessing the endocrine-disrupting effects of chemicals. We observed that gene transcripts (*tsh β* and *gpha*) encoding thyrotropin in the pituitary gland were TH regulated as part of a negative feedback loop. Our findings also show that transcripts for *tr α* and *tr β* are T₃ induced in the brain, and provide preliminary evidence that *bteb* transcription may be T₃ regulated in neural tissues of teleosts as it is in amphibians and mammals. Transcripts for *tr α* and *tr β* were similarly upregulated by T₃ in the liver of both male and female minnows, while only *tr β* was T₃ regulated in the gonads. Taken together, these results provide new insights into TH regulation of TR gene transcripts in target tissues of adult teleost fishes, while also identifying transcripts that may have application as endpoints for evaluating pollutant-induced disruption of the HPT axis in teleost models.

Declaration of interest

The authors declare that they have no conflict of interest that would prejudice the impartiality of results presented in this manuscript.

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