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Tissue distribution and thyroid hormone effects on mRNA abundance for membrane transporters Mct8, Mct10, and organic anion-transporting polypeptides (Oatps) in a teleost fish

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Abstract

Many of the actions of thyroid hormones (THs) occur via TH binding to intracellular receptors. Although it was long thought that THs diffused passively across plasma membranes, it is now recognized that cellular entry is mediated by a variety of membrane transporter proteins. In this study, we identified cDNAs encoding the TH transporters monocarboxylate transferase 8 (*mct8*) and 10 (*mct10*) as well as eight distinct organic anion-transporting polypeptide (*oatp*) proteins from fathead minnow (*Pimephales promelas*). Analysis of the tissue distribution of transporter mRNAs revealed that *mct8* and *mct10* transcripts were both abundant in liver, but also present at lower levels in brain, gonad and other tissues. Transcripts encoding *oatp1c1* were highly abundant in brain, liver and gonad, and exhibited significant sex differences in the liver and gonad. Treatment of adult male minnows with 3,5,3′-triiodothyronine (T_3) or the goitrogen methimazole altered gene transcript abundance for several transporters. Fish given exogenous T_3 had reduced *mct8* and *oapt1c1* mRNA levels in the liver compared to methimazole-treated fish. In the brain, transcripts for *mct8*, *mct10*, *oatp2b1*, and *oatp3a1* were each reduced in abundance in fish with elevated T_3. As a whole, these results provide evidence that TH status influences the transcriptional dynamics of *mct8*, *mct10* and several Oatp genes including *oatp1c1* in teleost fish.

Keywords

monocarboxylate transporter; Mct; gene expression; OATP; plasma membrane; solute carrier organic anion; liver; brain; transport protein; SLC16; *Pimephales promelas*

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Phylogenetics trees (Figs. 1 and 4) also available in interactive format

1. Introduction

Thyroid hormones regulate a variety of physiological and behavioral functions in teleost fishes including growth and morphological development (Reddy and Lam, 1992; Power et al., 2001; Lema and Nevitt, 2006), reproduction (Jacob et al., 2005; Nelson and Habibi, 2006), and behaviors such as rheotaxis and olfactory imprinting (McCormick et al., 1998; Lema and Nevitt, 2004; Edeline et al., 2005). These diverse functions are regulated in part by variation in the secretion of THs from the thyroid follicles, but also via extrathyroidal conversion of circulating thyroxine (T_4) either to the more bioactive 3,5,3′-triiodothyronine (T_3) or to less active forms (e.g., rT_3, T_2) by iodothyroinine deiodinase enzymes in peripheral tissues (Orozco et al., 2012). These peripheral tissues are also the sites where THs induce cellular responses, either by binding to intracellular thyroid hormones receptors (TRs) that function as transcription factors to activate or repress gene transcription, or via interactions with structural membrane proteins (e.g., integrin $\alpha v\beta 3$) to have non-genomic cellular actions (Bergh et al., 2005; Yonkers and Ribera, 2009; Cheng et al., 2010).

In mammals, it has recently become clear that the peripheral actions of THs also rely upon the presence of plasma membrane transporters expressed by a target cell or tissue (Bernal, 2005; Visser et al., 2011). Given the lipophilic properties of THs, it was long assumed that THs diffused passively across the lipid bilayer of plasma membranes (e.g., Robbins and Rall, 1960). It is now recognized, however, that transmembrane movement of THs is facilitated by several transporters belonging to the solute carrier (Slc) group of proteins, which includes the monocarboxylate transporter (Mct) and organic anion transporter polypeptide (Oatp) families of plasma membrane transporters (for reviews, see Bernal, 2005; Schweizer et al., 2008; Visser et al., 2011).

Of the Mct family, current evidence suggests that only Mct8 (solute carrier family 16 member 2, or Scl16a2) and Mct10 (solute carrier family 16 member 10, or Scl16a10) appear to function in the transport of THs (Halestrap & Meredith 2004, Visser et al., 2011), while other proteins in the Mct transporter family function in the movement of substrates such as aromatic amino acids, lactate, pyruvate, or ketone bodies across plasma membranes (Halestrap & Price 1999, Halestrap & Meredith 2004). Mct8 proteins from both human and rat show a high specificity for THs and have been documented to transport T_4, T_3, rT_3, and T_2 (Friesema et al., 2003; 2006; Visser et al., 2007). A recent study of the transport kinetics of zebrafish (*Danio rerio*) Mct8 indicated that this transporter likewise functions in TH transport, although the functional specificity of this fish protein may vary from that in mammals (Arjona et al., 2011). While not yet studied in any teleost fish, human Mct10 transports both T_3 and T_4, shows a greater affinity for T_3, and transports aromatic amino acids in addition to THs (Friesema et al., 2008; Visser and Visser, 2013).

The Oatp superfamily of transmembrane proteins mediates sodium-independent cellular transport of a wide variety of amphipathic organic compounds including xenobiotics, hormones, hormonal conjugates, bile salts, and pharmaceutical drugs (e.g., Hagenbuch and Meier, 2003; 2004; Mikkaichi et al., 2004; Hagenbuch and Gui, 2008; Svoboda et al., 2011). While over 160 Oatps have been identified to date from the sequenced genomes of more than 25 species (Hagenbuch and Gui, 2008), many basic questions remain unaddressed

about the tissue distribution, expressional regulation and functional differentiation of Oatps, even in humans (Svoboda et al., 2011). It is known, however, that select Oatps function in the transport of hormones or hormone metabolites including T_3, T_4, and glucuronidated steroid hormones (Pizzagalli et al., 2002; Hagenbuch, 2007; Hagenbuch and Gui, 2008), and recent studies in vertebrates have demonstrated a role for Oatps in hormone-associated contexts ranging from compensating for the endocrine disrupting effects of environmental pollutants (Szabo et al., 2009; Noyes et al., 2013), to changing their expression levels with hormone-dependent cancers (Pressler et al., 2011).

Mammalian Oatp1c1 (also known as Slco1c1 and Oatp14) has a high transport specificity for THs including T_3, T_4, rT_3, and T_4 sulfate (Sugiyama et al., 2003; Tohyama et al., 2004; Hagenbuch, 2007; van der Deure et al., 2008; Visser et al., 2011). Other mammalian Oatps including Oatp2b1, Oatp3a1, and Oapt4a1 also transport T_4 and, in some cases, T_3 or rT_3, although often with lower affinity (Fujiwara et al., 2001; Huber et al., 2007; van der Deure et al., 2008; for review, see Visser et al., 2011). It is thus likely that several Oatps contribute to the membrane transport of THs. In teleost fishes, however, almost nothing is known about evolutionary diversity and functional specificity of Oatp transporter proteins. The sole exception to date is one recent study in zebrafish that identified 14 *oatp* genes belonging to the eight families of Oatp proteins present in tetrapod vertebrates (Popovic et al., 2010), indicating that Oatp transporters can be more diverse in teleost fishes than mammals.

In this study, we identified cDNAs encoding Mct8, Mct10 and eight distinct Oatps from the fathead minnow (*Pimephales promelas*), a teleost fish commonly employed for studies on the toxic and endocrine disrupting effects of chemicals (Ankley and Villeneuve, 2006). Using real-time quantitative reverse transcription PCR (qRT-PCR) methods, we document the tissue distribution patterns for transcripts encoding these Mct and Oatp transporter proteins and report on relative expressional differences between sexes. We next explored whether circulating thyroid hormone status influences the relative abundance of *mct8*, *mct10*, and *oatp* transcripts in the brain and liver by experimentally modulating circulating TH levels in adult male minnows via oral treatment with exogenous T_3 or the goitrogen methimazole. In fish, as in mammals, the liver is the major organ of TH conversion and metabolism and therefore plays a key role in regulating systemic TH levels and effects (e.g., Van der Geyten et al., 1998; Malik and Hodgson, 2002). In the brain and nervous system of vertebrates, THs mediate cellular proliferation and neural differentiation (e.g., Forrest et al., 1996; Lema and Nevitt, 2004; Desouza et al., 2005), and limited evidence from the study of TH transporters in mammalian system indicates that TH status regulates mRNA and protein expression of Oatp1c1 in brain capillary endothelial cells (Sugiyama et al., 2003). We therefore hypothesized that circulating TH status would likewise influence the transcriptional dynamics for *oatp1c1*, and possibly for *mct8* and *mct10*, in the liver and brain of this teleost fish model.

2. Materials and Methods

2.1. Isolation and sequencing of mct and oatp cDNAs

2.1.1. Total RNA isolation—Liver tissue was dissected from an adult male fathead minnow (*Pimephales promelas*) purchased from Aquatic BioSystems, Inc. (Fort Collins,

CO, USA). The tissue was flash frozen in liquid N_2 and stored at −80 °C. Total RNA was extracted using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) with bromochloropropane for phase separation. The resulting RNA was DNase I treated (DNAfree Kit, Ambion) and quantified by spectrophotometery (Nanodrop 2000, ThermoScientific).

First stand cDNA was generated in 20 μL reverse transcription reactions by incubating 5 μg of total RNA template (4.0 μL) with 1.0 μL random primers (random hexadeoxynucleotides; Promega Corp., Madison, WI, USA) at 70 °C for 10 min. Subsequently, the above mixture was combined with 3.0 μL of $MgCl_2$ (25 mM), 1.0 μL deoxynucleotides triphosphates (dNTPs) (100 mM, Promega), 0.25 μL recombinant RNasin® ribonuclease inhibitor (20 U/μL, Promega), 4.0 μL of 5× reaction buffer, 0.5 μL GoScript™ reverse transcriptase, and 5.5 μL of nuclease-free H_2O, as per the protocol of the GoScript™ Reverse Transcription System (Promega). RNA was reverse transcribed under a thermal profile of 25 °C for 5 min and 42 °C for 1 h, followed by 70 °C for 15 min to inactivate the reverse transcriptase enzyme.

2.1.2. Isolation and sequencing of partial cDNAs encoding *mct8* and *mct10*—

In silico BLAST searches using mammalian *mct8* cDNA sequences identified ESTs encoding putative partial *mct8* cDNAs from Chinese rare minnow (*Gobiocypris rarus*; GenBank accession no. **EE393294**), zebrafish (**EH490251**), and three-spined stickleback (*Gasterosteus aculeatus*, DT965355), as well as an unannotated protein sequence from pufferfish (*Tetraodon nigroviridis*, **CAG09736**). Nucleotide sequences from the ESTs were aligned, and a nested set of degenerate primers was designed to consensus regions of these aligned sequences. Nucleotide sequences for these degenerate primers were as follows: (forward1) 5'-CTGCTACATGGTGCAACGG-3', (forward2) 5'-ACATGATGCTGGTGAARSACC-3', (reverse3) 5'-AAATAATGMCCCAGGATCACCAGG -3', (reverse2) 5'-GGCTGRAAYGCAAAYGAGGA-3', and (reverse1) 5'-GAGGAGCCGCAGCCRAASA -3' (Integrated DNA Technologies, Inc., Coralville, IA, USA). These degenerate primers were then used in 50 μL PCR reactions containing 25 μL of GoTaq® Polymerase Colorless Master Mix (Promega), 21 μL nuclease-free H_2O, 1 μL each of forward and reverse primers (50 μM), and 2 μL of cDNA. PCR products were examined on 1.2% agarose gels with EtBr, and any products of predicted size were cleaned (QIAQuick PCR Kit, Qiagen) and Sanger sequenced (MacrogenUSA, Rockville, MD, USA).

A sequence encoding *mct10* from zebrafish (*Danio rerio*, GenBank accession no. NM_001080028) was located in GenBank (http://www.ncbi.nlm.nih.gov/) and used to BLAST search EST and genomic databases (www.ensemble.org) for additional sequences from teleost fishes. These *in silico* searches identified an EST encoding a partial cDNAs for *mct10* from fathead minnow (**DT292022**), as well as unannotated ESTs encoding partial *mct10* cDNAs from three-spined stickleback (**DN712990**), Atlantic salmon (*Salmo salar*, **DY715060** and **AM402521**) and mandarin fish (*Siniperca chuatsi*, **GR474247**). An EST was also identified encoding *mct10* from Japanese ricefish (medaka, *Oryzias latipes*, **DK098183**), which was combined with several genomic sequences for *mct10* in this same species obtained from Ensemble. Resulting sequences were aligned using Sequencher v4

software (GeneCodes Corp., Ann Arbor, MI, USA), and gene-specific primers were designed to consensus regions and used to amplify a 615-bp nucleotide partial cDNA of *mct10* from fathead minnow. These primers were the following: (forward1) 5'-TCCAGAACGCCTTCGGTATCATGT-3', (forward2) 5'-TTCTTCTGCTCTCCGATCGTCAGT-3', and (reverse) 5'-AAGGCCCATATGCGGTATCCAAGA-3' (Integrated DNA Technologies, Inc.).

2.1.3. Identification of *oatp* partial cDNA sequences—Deduced protein sequences from previously identified *oatp* cDNAs from zebrafish (Popovic et al., 2010) were used for *in silico* BLAST searches to identify EST sequences encoding putative *oatp* cDNAs from fathead minnow. EST's identified from these searches were checked against zebrafish sequences to determine open reading frame regions, and then aligned using Sequencher v4 software (GeneCodes Corp.). Partial cDNAs encoding eight Oatps were identified from fathead minnow using this method: *oatp1c1* (GenBank accession nos. **DT233541** and **DT235267**), *oatp1f1* (**DT350917**), *oatp1f2* (**DT350916**), *oatp2a1* (**DT161775**), *oatp2b1* (**DT195991**), *oatp3a1* (**DT287125**), *oatp4a1* (**DT228118, DT173660** and **DT118223**), and *oatp5a1* (**DT197132**). Nested sets of gene-specific primers were designed for each of these eight *oatp* cDNAs, and used to amplify and sequence each *oatp* cDNA to confirm nucleotide composition. Nucleotide sequences for these primers are provided in Table 1 of the Online Supplemental Materials. Thermal cycling conditions for the PCR reactions were as follows: 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 1–2 min, and then 72 °C for 2–4 min, followed by a second round of PCR using 95 °C for 2 min, 30 cycles of 95 °C of 30 s, 55 °C for 30 s, and 72 °C for 1–2 min, and then a final extension step of 72 °C for 3–4 min. All PCR products were examined on 1.2% agarose gels, and the resulting PCR products were purified (QIAQuick PCR Kit, Qiagen) and Sanger sequenced (MacrogenUSA).

2.2. Phylogenetic analyses

Deduced amino acid sequences obtained from fathead minnow *mct* and *oatp* cDNAs were aligned to all teleost fish sequences of Mct8 and Mct10 proteins or Oatp proteins identifiable on GenBank, as well as select tetrapod sequences. Amino acid sequences for Mct8 and Mct10 were aligned using ClustalX software (Larkin et al., 2007), and an unrooted tree was assembled by the Neighbor-Joining method (Saitou and Nei, 1987) using the p-distance model with pairwise deletion of gaps in MEGA v5 software (Tamura et al., 2011). Confidence values for nodes were determined using bootstrap tests (1000 replicates). For the Oatp phylogeny, a tree was constructed by the maximum likelihood method using the Jones-Taylor-Thornton model (Jones et al., 1992) with all amino acid sites included. Again, 1000 bootstrap replicates were used to determine confidence values for each node.

2.3. Tissue distribution of relative mRNA abundance

Adult fathead minnows (*Pimephales promelas*) were purchased from Aquatic BioSystems, Inc. (Fort Collins, CO, USA), and maintained in closed system, 208 L tanks under conditions of 24–26 °C, 0.2 ppt salinity, and a 14L:10D photoperiod. Fish were maintained for 30 days prior to commencing any fish sampling. All procedures were approved by the

Animal Care and Use Committee of California Polytechnic State University (Protocol no. 1207).

Adult male (n = 3; body mass, 3.96 ± 0.69 g [mean ± SEM]; standard length, 53.87 ± 3.45 mm) and female (n = 3; 1.74 ± 0.24 g; 41.07 ± 2.24 mm) minnows were euthanized in buffered tricaine methansulfonate (MS222), and the brain, gills, gonads, liver, intestine, spleen, kidney, skeletal muscle were dissected. The brain was further subdivided into the forebrain, hypothalamic, optic tectum, cerebellum and hindbrain (medulla oblongata). All tissues were immediately frozen in liquid N_2 and stored at −80 °C.

Total RNA was extracted using TriReagent (Molecular Research Center, Inc.), DNase I treated (TURBO DNAfree Kit, Ambion), and quantified using a P300 NanoPhotometer (Implen, Inc., Westlake Village, CA, USA). Total RNA was then reverse transcribed in 30 μL reactions by incubating 15.5 μL of total RNA template (7 μg) with 6.0 μL 5× buffer, 0.25 μL of nuclease-free H_2O (Sigma-Aldrich, St. Louis, MO, USA), 4.5 μL $MgCl_2$, 1.5 μL dNTPs, 1.0 μL of random primers, 1.0 μL of GoScript™ reverse transcriptase, and 0.25 μL of RNasin® ribonuclease inhibitor (20 U/μL, Promega). The mixture was incubated under a thermal profile of 25 °C for 5 min followed by 42 °C for 60 min and 70 °C for 15 min (T100 thermal cycler, BioRad).

Primers and Taqman probes for quantitative real-time PCR assays were designed to the cDNAs encoding *mct8*, *mct10*, and the eight *oatps* from fathead minnow. Nucleotide sequences for these primers and probes are provided in Table 1. Quantitative real-time PCRs were conducted in 16 μL reactions, with each reaction containing 4.5 μL nuclease-free water (Sigma), 8.0 μL qPCR Master Mix (iTaq™ Universal SYBR® Green Supermix, BioRad), 0.5 μL of both forward and reverse primer (45 μM), 0.5 μL probe (5 μM), and 2.0 μL of cDNA template. The PCR thermal profile for each reaction was 50 °C for 2 min, 95 °C for 3 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min, and all assays were run on a 7300 Real Time PCR System (Applied Biosystems, Inc.). For each gene, a standard curve was made from a pool of total RNA from samples representing all treatments and sexes, and each standard was assayed in triplicate. DNA contamination was assessed for each gene by analyzing RNA samples that were not reverse-transcribed, and each qPCR run included two samples without cDNA template to further control for contamination. PCR efficiencies for each transcript assay are provided in Table 1, and correlation coefficients (r^2) for all transcripts were always greater than $r^2 = 0.991$. Finally, expression of each gene of interest was expressed as a relative level by dividing the resulting values by the mean values of the forebrain mRNA levels in each gene.

2.4. Effects of thyroid hormone status on *mct* and *oatp* mRNA levels

2.4.1. Dietary T_3 and methimazole treatments—Exogenous T_3 (Sigma-Aldrich) or the goitrogen methimazole (Sigma-Aldrich) were administered orally within a 1:1 ratio mixture of commercial spirulina flake (Aquatic Eco-Systems, Inc., Apopka, FL) and brine shrimp flake (San Francisco Bay Brand, Inc., Newark, CA, USA) feed. Flake food was sprayed with a 20 mL solution containing 19 mL of 95% ethanol and 1 mL of 0.1% NaOH containing either 20 mg T_3 (Sigma-Aldrich), 150 mg methimazole (Sigma-Aldrich), or no additional chemical (control).

Adult minnows (n = 16–18 per treatment; body mass: 3.25 ± 0.10 g; standard length: 52.19 ± 0.57 mm; mean ± SEM) were maintained in 113 l tanks under conditions of 25.80 ± 0.01 °C, and 0.2 ppt salinity. Fish were fed 0.05 g flake feed · $fish^{-1}$ · day^{-1}, over two separate feeding times (11:00–13:00 h and 16:00–19:00 h), for average exposure doses of 20 μg T_3 · $fish^{-1}$ · day^{-1} and 150 μg methimazole · $fish^{-1}$ · day^{-1}. After 14 days of treatment, minnows were euthanized (MS222) and body mass (g) and standard length (mm) were recorded. Blood was collected in heparinized capillary tubes from the caudal peduncle, transferred to heparinize microcentrifuge tubes, and centrifuged at 3,000 *g* for 10 min at 4 °C, and the resulting plasma was stored at −80 °C for subsequent quantification of plasma TH concentrations. The whole brain and liver were dissected, frozen in liquid N_2, and then stored at −80 °C.

2.4.2. Quantification of plasma total T_4 and T_3 concentrations—Plasma from individual male fish of the same treatment tank was pooled (*n* = 3–6 fish per pool), and circulating total T_4 (tT_4) and total T_3 (tT_3) concentrations were quantified by liquid chromatography tandem mass spectrometry (LC/MS/MS) (Wang and Stapleton, 2010) in conjunction with a newly developed extraction method for fish plasma described in detail elsewhere (Noyes, 2013; Noyes et al., submitted). Briefly, THs were extracted by incubating the pooled plasma samples (50 μl) with 50 μl of isotopically labeled hormones $^{13}C_{12}$-T_4 and $^{13}C_6$-T3 (10 ng/mL in MeOH; Cambridge Isotope Laboratories, Andover, MA), which were used as internal standards to quantify levels of tT_4 and tT_3, respectively. Blank controls (deionized water) containing labeled THs alone were extracted alongside plasma samples to correct for trace unlabeled hormone levels (~0.5%) present in the labeled standards. The labeled hormone $^{13}C_6$-T_4 (Isotec, Canton, GA) was added prior to LC/MS/MS analysis to measure recovery of the $^{13}C_{12}$-T_4 internal standard. The percent recovery of $^{13}C_{12}$-T_4 was 93.50 ± 6.85 % (mean ± SD). Standard curves for each hormone spanned from 0.05 to 10 ng/mL with r^2 values for each curve using a quadratic regression at > 0.99. Method detection limits (MDLs) were defined as three times the standard deviation (mean + 3*SD) of thyroid hormones detected in blanks containing no plasma and were measured at 0.25 ng/mL and 0.16 ng/mL for tT_4 and tT_3, respectively. The resulting TH values were normalized to the volume of plasma extracted (50 μL). The average intra-assay CV % for this LC/MS/MS method with fathead minnow plasma was calculated to be 4.73% for tT_3 and 5.97% for tT_4 (Noyes et al., submitted).

2.4.3. Quantitative real-time RT-PCR—Total RNA was extracted using TriReagent (Molecular Research Center, Inc.), DNase I treated (TURBO DNAfree Kit, Ambion), and quantified (P300 NanoPhotometer; Implen, Inc.). Total RNA was then reverse transcribed in 30 μL reactions by incubating 15.5 μL of total RNA template (10 μg/μL) with 6.0 μL 5× buffer, 0.25 μL of nuclease-free H_2O (Sigma-Aldrich), 4.5 μL $MgCl_2$, 1.5 μL dNTPs, 1.0 μL of random primers, 1.0 μL of GoScript™ reverse transcriptase, and 0.25 μL of RNasin® ribonuclease inhibitor (20 U/μL, Promega). The mixture was incubated under a thermal profile of 25 °C for 5 min followed by 42 °C for 60 min and 70 °C for 15 min (T100 thermal cycler, BioRad).

Quantitative real-time PCRs were conducted in 16 μL reactions as described above. Nucleotide sequences for primers and probes are shown with average PCR efficiencies for each transcript in Table 1, and r^2 for all standard curves were greater than $r^2 = 0.97$. Transcript abundance for ribosomal protein L8 (*rpl8*) was also quantified for use as the normalizing gene in liver, while elongation factor 1α (*ef1*α) was used as the normalizing gene in the brain. These transcripts were selected for each tissue because their relative expression levels did not vary across treatment groups (liver *rpl8*, $F_{2,52} = 0.013$, $p = 0.987$; brain *ef1*α, $F_{2,47} = 0.898$, $p = 0.414$). For each gene, relative mRNA levels were subsequently calculated using the standard curve and normalized to *rpl8* mRNA expression (liver) or *ef1*α mRNA expression (brain). Finally, expression of each gene of interest was expressed as a relative level by dividing the resulting values by the mean values of the forebrain mRNA levels in each gene.

2.5. Statistical analyses

Tissue distribution data on relative levels of *mct* and *oatp* mRNAs was square root transformed to equalize variances. Two-tailed *t* tests with modified α levels as per the step-down Bonferroni-Holm correction method (Holm, 1979) were then used to examine sex differences in transcript abundance for each transcript within each tissue. This approach of using *t* tests in combination with step-down modified α levels was chosen to balance our reporting of tissues where clear sex differences in *mct* or *oatp* transcript abundance appeared evident in the data with the family-wise type I statistical error rate, given the large number of tissues and transcripts examined.

Plasma TH concentrations in male minnows treated with feeds containing exogenous T_3, the goitrogen methimazole, or control vehicle were analyzed using a one-factor ANOVA model followed by Tukey HSD multiple comparisons. Analysis of relative levels of each *mct* and *oatp* transcript in the liver and brain was likewise conducted using a one-factor ANOVA model followed by Tukey HSD multiple comparison tests ($\alpha = 0.05$). For select transcripts, data were square root transformed to homogenize variances prior to running ANOVA models. All data are presented as mean ± SEM.

3. Results

3.1. Sequencing and tissue distribution of mct8 and mct10 cDNAs

Two partial cDNAs encoding putative *mct8* (231 bp nucleotides, GenBank accession no. **KF053157**) and *mct10* (846 bp nucleotides; **KF053158**) were identified from fathead minnow by RT-PCR using degenerate primers. BLAST analyses (http://blast.ncbi.nlm.nih.gov) confirmed that these two cDNA fragments shared both nucleotide and deduced amino acid sequence identity with mammalian *mct8* and *mct10* genes, as well as the previously identified *mct10* cDNA from zebrafish (**NM_001080028**). Alignment and phylogenetic analysis of the predicted amino acid sequences of these fathead minnow *mct8* and *mct10* cDNAs further confirmed their identities as *mct8* and *mct10* (Fig. 1).

The relative abundance of *mct8* and *mct10* mRNAs, as indicated by quantitative real-time RT-PCR, was found to vary among tissues (Fig. 2). Both *mct8* and *mct10* mRNAs were at

greatest abundance in the liver, although *mct10* transcripts also showed a high abundance in kidney and, to a lesser degree, gonad tissues. The abundance of *mct10* transcripts in the liver was observed to be at greater relative levels in males than in females ($t = 4.2502$, $p = 0.0196$) (Fig. 3). Sex differences in gene transcript abundance were also observed for *mct8* in the gonad, with higher *mct8* mRNA levels in ovarian tissues than in testicular tissues ($t = -5.1840$, $p = 0.0226$) (Fig. 3).

3.2. Phylogenetic analysis and tissue distribution of *oatp* mRNAs

BLAST (http://blast.ncbi.nlm.nih.gov) analyses of available unannotated EST sequences from fathead minnow identified eight putative *oatp* cDNAs. Gene-specific primers were subsequently designed to each of these cDNAs and used in PCRs to amplify and sequence each cDNA to confirm nucleotide sequence composition. The resulting cDNAs included the following: a partial cDNA of 870 bp nucleotide encoding *oatp1c1* (slco1c1; GenBank accession no. **FK053149**), two partial cDNAs of 589 bp and 311 bp nucleotides encoding putative Oatp1f-type transporters *oatp1f1* (slco1f1; **FK053150**) and *oatp1f2* (slco1f2; **FK053151**), a partial *oatp2a1* cDNA of 422 bp nucleotides (slco2a1; **FK053152**), a 700 bp nucleotide partial cDNA encoding *oatp2b1* (slco2b1; **FK053153**), a partial *oatp3a1* cDNA of 464 bp nucleotides (slco3a1; **FK053154**), a partial cDNA of 1,462 bp nucleotides encoding *oatp4a1* (slco4a1; **FK053155**), and a partial cDNA of 618 bp nucleotides encoding *oatp5a1* (slco5a1; **FK053156**). Phylogenetic analysis of the predicted amino acid sequences of each of these *oatp* cDNAs from fathead minnow revealed that each cDNA grouped into a separate clade of Oatp genes, with the exception of the *oatp1f1* and *oatp1f2* cDNAs, which grouped within a single Oatp1f-type clade (Fig. 4). Four distinct full-length Oatp1f-type cDNAs were previously identified from zebrafish, indicating that some Cyprinid fishes may have multiple Oatp1f-type transporters evolved through gene duplication (Popvic et al., 2010).

Transcripts encoding *oatp* anion transporter proteins differed in relative abundance among organs and tissues (Fig. 5). Relative transcript levels of *oatp1c1* were greatest in the gonad, liver and brain (Fig. 5A). The high relative abundance of *oatp1c1* mRNAs in the gonad appeared to be due to elevated levels of *oatp1c1* mRNAs in ovarian tissues, since *oatp1c1* mRNAs were on average more than 7 fold greater in the ovary than in the testis (Fig. 6B; $t = -12.395$, $p = 0.0007$). A sex difference in *oatp1c1* transcript abundance was also observed in the liver, with male minnows exhibiting 4-fold greater *oatp1c1* mRNA levels in this tissue compared to females (Fig. 6A; $t = 5.331$, $p = 0.0064$).

Relative levels of *oatp1f1* and *oatp1f2* were observed to be greatest in the kidney (Fig. 5B,C). Transcripts encoding *oatp2a1* were most abundant in brain, gill, gonad and liver (Fig. 5D), and while *oapt2b1* mRNAs were observed to be at greatest abundance in gill tissue, a significant sex difference in relative levels of *oapt2b1* was observed in the liver (Fig. 6A; $t = 3.693$, $p = 0.021$). Other Oatp-encoding mRNAs likewise exhibited expressional variation in relative abundance among tissues, with *oatp3a1* mRNAs most abundant in cardiac muscle (Fig. 5F), *oatp4a1* mRNAs most abundant in brain and gonadal tissues (Fig. 5G), and *oatp5a1* transcripts at greatest levels in brain, gonad and heart (Fig. 5H). Transcripts encoding *oatp5a1* were observed to be at greater relative levels in the liver

of male fish compared to females (Fig. 6A; t = 7.908, $p = 0.0015$), and both *oatp4a1* and *oatp5a1* transcripts were at higher abundance in ovarian tissue than in testicular tissue (Fig. 6B; *oapt4a1*, t = −6.363, $p = 0.0231$; *oatp5a1*, t = −7.632, $p = 0.0017$).

3.3. Thyroid hormone regulation of *mct* and *oatp* mRNAs in liver and brain

Oral treatment of adult male minnows with either exogenous T_3 or the goitrogen methimazole generated variation in plasma concentrations of total T_4 (tT_4) ($F_{2,15} = 5.255$, $p = 0.0186$), and *post hoc* pairwise comparisons revealed tT_4 to be significantly lower in methimazole-treated fish compared to T_3-treated fish (Fig. 7). Plasma concentrations of total T_3 (tT_3) also differed among treatment groups, and were significantly elevated in males treated orally with exogenous T_3 ($F_{2,15} = 291.35$, $p < 0.0001$) (Fig. 7).

These experimentally-induced changes in circulating THs were found to influence relative mRNA levels for select *mct* and *oatp* membrane transporter genes, with the specific effects varying depending on transcript and tissue. In the liver, male fish treated with exogenous T_3 had lower relative levels of *mct8* transcripts compared to methimazole-treated fish (Fig. 8A; $F_{2,52} = 3.839$, $p = 0.0279$). Experimental modulation of plasma TH levels did not, however, alter liver *mct10* mRNA levels (Fig. 8A). In the liver, transcripts encoding *oatp1c1* were found to be strongly altered by T_3 treatment, with T_3-exposed fish showing more than a 60% reduction in *oatp1c1* mRNA abundance compared to control or methimazole-treated fish (Fig. 8B; $F_{2,52} = 14.273$, $p < 0.0001$). Transcripts for *oatp2a1* were likewise reduced in abundance in the liver tissue of T_3-treated males (Fig. 8B; $F_{2,52} = 4.544$, $p = 0.0152$). The relative abundance of *oatp5a1* transcripts was also altered by the experimentally-induced change in plasma TH concentrations, although this effect differed qualitatively from that observed for *oatp1c1* and *oatp2b1* transcripts with methimazole-treated fish exhibiting elevated *oatp5a1* mRNA levels compared to control fish (Fig. 8B; $F_{2,52} = 4.549$, $p = 0.0151$).

In the brain, both *mct8* and *mct10* showed changes in relative mRNA abundance in response to experimental alteration of plasma TH concentrations. Minnows treated with T_3 had a reduced relative abundance of transcripts encoding *mct8* ($F_{2,47} = 6.829$, $p = 0.0025$) and *mct10* ($F_{2,47} = 3.819$, $p = 0.0291$) compared to methimazole-treated fish (Fig. 9A). Transcripts encoding *oatp2b1* and *oatp3a1* also exhibited small (< 10%), but statistically significant reductions in relative abundance in response to T_3-treatment (Fig. 9B), with *oatp2b1* mRNA levels lower in T_3-treated males than control males ($F_{2,47} = 3.346$, $p = 0.044$), and *oatp3a1* transcripts reduced in T_3-treated fish compared to methimazole-treated fish ($F_{2,47} = 3.504$, $p = 0.0381$).

4. Discussion

Only within the last decade or so has it become evident that the movement of THs into target cells occurs not by diffusion, but rather via facilitated transport mediated by plasma membrane transport proteins (Bernal, 2005; Visser et al., 2011). The first TH transport protein was identified in mammals at the molecular level over 15 years ago (Abe et al., 1998), and yet almost nothing is presently known about the diversity, patterns of expression, or regulation of TH membrane transporters in other vertebrates including teleost fishes. In

this study, we identified cDNAs encoding *mct8*, *mct10*, and eight transporters from the Oatp family of solute carrier proteins in the fathead minnow, and documented the expressional distribution of mRNAs encoding these transporters among organs and tissues. We also began to explore how varying conditions of thyroid hormone status may alter the expression of these transporters by experimentally modulating circulating TH status and then quantifying the effects on relative mRNA levels for these membrane transporters in the liver and brain.

4.1. Distribution of monocarboxylate transporter Mct8 and Mct10 transcripts

Using degenerate primer PCR and *in silico* searches of available EST libraries from teleost fishes, partial cDNA sequences encoding *mct8* and *mct10* from the fathead minnow were isolated and sequenced. Phylogenetic analysis of the deduced amino acid sequences from these cDNAs confirmed the identity of these mRNAs and revealed that each respective protein comprised part of clades containing previously identified *mct8* and *mct10* proteins from mammals, as well as *mct8* and *mct10* genes recently annotated from the genomes of select fish, birds and reptiles (e.g., zebrafish, *Takifugu* pufferfish, green anole) and other teleost fish cDNAs newly identified from unannotated EST libraries as part of the current study (Fig. 1). Quantitative real-time RT-PCR (qRT-PCR) analyses of the relative levels for *mct8* and *mct10* transcripts in fathead minnow tissues revealed that mRNAs for both genes are most abundant in the liver, but are also present at lesser abundances in all organs and tissues examined.

Mammalian Mct8 has likewise been detected in a variety of tissues including brain, liver, kidney, thyroid gland, placenta, and the adrenal glands (Price et al., 1998; Biebermann et al., 2005). Mammalian Mct8 has a high specificity for the bidirectional transport of a variety of THs (T_4, T_3, rT_3 and T_2) across cellular membranes (Friesema et al., 2003, 2006), but its role in regulating T_3 transport across the blood-brain barrier is perhaps the best evidence that Mct8 plays a key role in target tissue responses to circulating THs (reviewed by Visser et al., 2011). Clinical studies in humans have now linked several mutations in Mct8 to behavioral and cognitive abnormalities including impaired speech development, dystonic movements, low intelligence quotient values, and mental retardation (e.g., Dumitrescu et al., 2004; Schwartz and Stevenson, 2007; Visser et al., 2011; Visser and Visser, 2012). Clinical patients with non-functional Mct8 often also show atypical TH signaling profiles with elevated circulating T_3 and reduced total and free T_4 and rT_3 concentrations suggestive of compensatory responses in either TH secretion or peripheral conversion by iodothyronine deiodinases (Visser et al., 2011). Mammalian Mct10, the most structurally similar Mct protein to Mct8, has also been shown to transport both T_3 and T_4 (Friesema et al., 2008). Recently, Arjona and coworkers (2011) provided the first characterization of Mct8 from a teleost fish, the zebrafish *Danio rerio*, and demonstrated that zebrafish Mct8 (zMct8) likewise mediates cellular TH uptake. Similar to our findings here with fathead minnows, *mct8* mRNAs are found nearly ubiquitously in adult zebrafish tissues, with greatest expression levels in brain, liver and kidney (Arjona et al., 2011). Transcripts encoding Mct8 also are present in zebrafish during early developmental stages (Arjona et al., 2011), and morpholino-based knockdown and rescue of *mct8* in this species provides evidence that zMct8 expression during early life is required for brain and spinal cord development (Vatine

et al., 2013). Interestingly, however, the transport specificity of zMct8 appears to vary from mammalian Mct8 in that zMct8 transports T_3 with a higher affinity and also shows temperature-dependent variation in TH specificity, transporting only T_3 at a temperature of 26 °C, but both T_3 and T_4 at 37 °C (Arjona et al., 2011). An environmental temperature of 37 °C is much higher than standard laboratory rearing conditions for zebrafish and approaches the maximum recorded temperatures for this species in the wild (Engeszer et al., 2007). Nonetheless, many teleost fishes tolerate fluctuating water temperatures daily or seasonally, and future studies into the temperature dependency of Mct8 function in fish could provide new insights into the dynamics of TH signaling in variable temperature environments.

Our initial comparisons of *mct8* and *mct10* mRNA levels among male and female minnows also revealed evidence of sex differences in *mct* transcript abundance in select tissues. Relative transcript abundance for *mct10* in the liver was more than 2-fold greater in males than in females, and ovarian *mct8* mRNA levels were nearly 2-fold higher than testicular *mct8* mRNA levels (Fig. 3). For gonadal tissues, such differences in *mct8* mRNA levels are likely associated with differential roles for THs in ovarian and testicular function in fish. THs have been known to influence reproductive function in fish for some time (Cyr and Eales 1996), and there is evidence that thyroid status can affect testicular androgen production and spermatogenesis (Jacob et al., 2005; Swapna et al., 2006). In fathead minnows, ovarian and testicular tissues have previously been shown to vary in the expression of other TH signaling components such as TH receptors α and β. For instance, the relative abundance of TH receptor β transcripts in the testis is associated negatively with the proportion of primary and secondary spermatocytes, but positively with the proportion of spermatozoa (Lema et al., 2009). From this evidence it is clear that patterns of gene expression for proteins involved in TH signaling in the gonad will be highly dependent on variation in cellular composition with oogenesis and spermatogenesis, and additional studies focused on the role of THs in gonadal function in fish are needed. It is also important to note, however, that the small sample sizes of each sex used to assess tissue distributions in the present study points to a need for additional studies to confirm these sex differences in gonadal *mct8* mRNA expression. Future studies should use these results as a foundation to confirm and extend the study of sexual variation in Mct expression in teleost fish.

4.2. Diversity and distribution of Oatp transporters

The Oatp family of solute carrier proteins is highly diverse with most proteins in this family transporting a variety of ligands (Hagenbuch and Meier, 2003, 2004; Hagenbuch, 2007; Hagenbuch and Gui, 2008). From the fathead minnow, we identified eight putative *oatp* cDNAs from seven *oatp* gene families, including what appears to be two distinct *oatp1f* cDNAs (Fig. 4). These results provide only the second assessment of Oatp transporter diversity in a teleost fish, as previous work by Popovic and coworkers (2010) identified 14 distinct *oatp* genes in the zebrafish, including paralogs of *oatp3a* and *oatp5a*, and four distinct *oatp1f* genes. These findings in zebrafish point to the likehood of considerable variation in *oatp* diversity among teleost fish species, possibly in accordance with taxonomic variation in genome duplication among Actinopterygiian fishes (e.g., Larhammar et al., 2009).

Although only limited comparative data are available at present, the tissue distribution patterns of relative *oatp* mRNA abundance observed here in adult fathead minnow (Fig. 5) generally corresponds with the pattern observed previously in adult zebrafish (Popovic et al., 2010). Popovic et al. (2010) likewise found *oatp2b1* mRNAs to be highly abundant in the gills, and *oatp1f* transcript most abundant in kidney and at low or undetectable levels in other tissues. Popovic and coworkers (2010) did not, however, target *oatp1c1* in the zebrafish, so there is no data available to compare our finding of *oatp1c1* mRNAs as abundant in the brain, liver and gonadal tissues of adult fathead minnows. Distinct sex differences were also measured in relative *oatp1c1* expression with greater abundance in the liver of males than females (Fig. 6A) as well as elevated levels in the ovary compared to testis (Fig. 6B). Similar patterns of male-female transcript abundance variation were also observed for *oatp2b1* and *oatp5a1* in the liver and *oatp4a1* and *oatp5a1* in the gonadal tissues (Fig. 6).

Although beyond the scope of the current study, it is not expected that all Oatps identified here function in the membrane transport of THs in fathead minnows as the functional specificity for substrate transport varies widely among mammalian Oatps (e.g., Hagenbuch and Meier, 2003; Meier-Abt et al., 2006; Hagenbuch, 2007; Svoboda et al., 2011). Rather, several of these Oatps have established alternate functions in mammals; mammalian Oatp2a1, for instance, is a key prostaglandin transporter, and Oatp2b1 is involved in the liver uptake and disposition of several classes of xenobiotics. It is still largely unclear which compounds Oatp5a1 transports, even in mammals (Svoboda et al., 2011). Of the eight *oatp* cDNAs identified here in the fathead minnow, only *oatp1c1*, *oatp2b1*, *oatp3a1* and *oatp4a1* have been identified as TH membrane transporters in mammalians systems (Mikkaichi et al., 2004; Svoboda et al., 2011; Visser et al., 2011).

Of this group of TH transporting Oatps, mammalian Oatp1c1 has the highest affinity for THs (T_4, T_3, rT_3) (Pizzagalli et al., 2002), although this protein also is involved in the uptake of estrone-3- sulfate and 17β-estradiol-glucuronide (Sugiyama et al., 2003; Hagenbuch, 2007; van der Deure et al., 2008; Visser et al., 2011). In mammalian systems, Oatp1c1 appears to play key roles in TH uptake across the blood-brain barrier as well as by Leydig cells in the testis (Sugiyama et al., 2003; Tohyama et al., 2004; Chu e al., 2008). Our finding of high *oatp1c1* mRNA abundance in the brain, gonad and liver may therefore support a similar important of Oatp1c1 in these tissues in fish. In any case, much remains unknown about the function and expressional dynamics of Oatp1c1, even in mammals. For instance, human patients identified with genetic mutations in *oatp1c1* continue to have circulating THs in the normal physiological range (van der Deure et al., 2008). And yet, experimentally-induced changes in circulating TH concentrations have been observed to alter Oatp1c1 protein and mRNA levels in endothelial cells in the rat brain (Sugiyama et al., 2003), suggesting that systemic THs may regulate Oatp expression under some circumstances.

4.3. Effects of circulating TH concentrations on *mct* and *oatp* mRNA levels

Regulation of TH signaling is known to occur not only via changes in the production and secretion of THs from the thyroid follicles, but also via the up- or down-regulation of

iodothyronine deiodinase enzymes or the abundance and type of thyroid hormone receptors in peripheral tissues (Cheng et al, 2010; Orozco et al., 2012). Such peripheral regulation can generate variation among target tissues in sensitivity to circulating hormones (e.g., Lema and Kitano, 2013). Target tissue variation in the type and density of TH membrane transporters may therefore provide an additional mechanism for regulatory control of TH action on peripheral tissues. At present, however, our understanding of TH transporter regulation is limited, and to our knowledge only a few studies have tested whether TH membrane transporters show expressional regulation under controlled conditions where TH titers are modulated experimentally. In one recent study of male New Zealand rabbits manipulated to generate a phenotypic state mimicking illness, 'ill' rabbits were found to have elevated mRNA levels of *mct8* in liver and *mct10* in skeletal muscle; treatment of these rabbits with a combination of exogenous T_4 and T_3 was observed to down-regulate *mct8* gene transcription in liver and *mct10* transcription in muscle (Mebis et al., 2009). And, in a separate study of rats, hypothyroidism caused either by thyroidectomy or by treatment with the goitrogen methimazole was found to increase *oatp1c1* mRNA levels and protein levels in the endothelial cells lining brain capillaries; exogenous T_3-induced hyperthyroidism was observed to have the opposing effect in these same animals (Sugiyama et al., 2003). And, in a recent study of adult fathead minnows similar to our work here, oral dosing of males with either the goitrogen 6-propyl-2-thiouracil (PTU) or the (PBDE) flame retardant decabromodiphenyl ether BDE-209 both resulted in an increase in brain *mct8* mRNA levels (Noyes et al., 2013).

In the present study, we tested whether changes in systemic TH status would alter gene transcript abundance for TH transporters in fish tissues by exposing adult male minnows orally for 14 days to either exogenous T_3 or methimazole, and then quantifying relative *mct* and *oatp* mRNA levels in the liver and whole brain. In the liver, fish with experimentally elevated plasma T_3 levels showed a nearly 70% reduction in relative transcript abundance for *oatp1c1*, and also induced smaller reductions in *mct8* and *oatp2a1* mRNA levels. In the brain, fish treated with exogenous T_3 showed reduced abundances of *mct8*, *mct10* and *oatp3a1* compared to methimazole-treated fish. Transcripts encoding *oatp2b1* in the brain also varied among treatment groups, with T_3-treated fish exhibiting reduced *oatp2b1* mRNA levels relative to control fish. Interestingly, these *oatp* transporter transcripts that we observed responding to T_3 or goitrogen treatment correspond with Oatps identified as transporting THs in mammalian systems (e.g., Oatp1c1, Oatp3a1, and Oatp2b1) with the exception of Oatp2a1, which transports prostaglandins in mammals, and Oatp5a1, which has no identified substrates to date (Hagenbuch, 2007; Svoboda et al., 2011).

The direction of abundance changes that we observed in *mct* or *oatp* mRNAs generally follows the pattern predicted if these changes were part of a compensatory response, where higher circulating T_3 concentrations down-regulate TH transporter expression to reduce TH uptake, and lower circulating THs trigger an up-regulation of transporter gene transcription. With the exception of *oatp1c1* down-regulation in the liver of T_3-treated fish, however, all other changes in membrane transporter mRNA abundance that we observed varied no more than 15–20% from the levels observed in control or methimazole-treated fish, even under the current experimental scenario where plasma tT_3 concentrations exceeded the normal

physiological range for this species (e.g., Lema et al., 2008; 2009). This finding may suggest that transcriptional responses of TH transporters to alterations in circulating TH concentrations may be a fairly minor component in the regulation of peripheral tissue sensitivity compared to TH-induced changes in deiodinase or TH receptor expression (Johnson and Lema, 2011). Furthermore, evidence from mutant and gene knockout studies in mammals suggests that changes in TH membrane transporter density itself may trigger compensatory changes in deiodinase enzymes or TH receptor density, as target cells counteract a reduced capacity to uptake THs (e.g., Dumitrescu et al., 2006; Mayerl et al., 2012).

While more studies are needed to understanding the dynamics of TH membrane transporter regulation in modulating tissue sensitivity to THs, our results here indicate that changes in TH transporter gene transcription in response to TH can vary among tissues. Even though our analysis of *mct* and *oatp* mRNA tissue distributions indicated that *oatp1c1* transcripts are abundant in both brain and liver tissues (Fig. 5A), the strong down-regulation of *oatp1c1* mRNAs in the liver – but absence of an *oapt1c1* transcriptional response in the brain – of T_3-treated minnows provides evidence for differences in TH transport regulation in these tissues. Supporting this finding, we recently observed that oral treatment of adult male fathead minnows with the goitrogen PTU induced a 12-fold increased in *oatp1c1* transcript abundance in the liver, but had no effect on *oatp1c1* mRNA levels in the brain (Noyes et al., 2013). In this same study, dietary exposure of male minnows to the PBDE flame retardant BDE-209 depressed circulating tT_3 and tT_4 concentrations and increased liver *oatp1c1* mRNA levels nearly 7-fold, but again had no effect on brain *oatp1c1* transcript abundance (Noyes et al., 2013). While the functional significance of tissue variation in *oatp1c1* transcriptional dynamics remains unclear, the absence of a detectable response of *oatp1c1* mRNAs in the brain may indicate a lessened ability for this tissue to modulate TH uptake (e.g., Lema et al., 2008; Noyes et al., 2013). Under normal conditions, the liver is thought to be the primary organ for conversion and metabolism of THs in blood circulation (Van der Geyten et al., 1998; Malik and Hodgson, 2002). Here we found that the effects of plasma TH concentrations on liver *oatp1c1* transporter mRNA abundance were particularly large in magnitude, suggesting that regulatory changes in liver Oatp1c1 expression may be a key component in maintaining systemic TH homeostasis in teleost fishes. Even so, large changes in circulating TH concentrations induced either experimentally or via exposure to EDC pollutants could overwhelm the ability of the liver to adjust circulating THs, so that the brain's ability to regulate TH uptake may be key in determining the impacts of altered TH status on brain development and function (Zoeller et al., 2002; Tan and Zoeller 2007). It is also important to note that the absence of *oatp1c1* mRNA changes in the brain of T_3-treated fish stands in contrast to the regulatory response of *oatp1c1* mRNAs and protein observed in the brain capillary endothelial cells of rats (Sugiyama et al., 2003). This distinction may reflect the differences in TH transport mechanisms across the blood-brain barrier between mammals and fish, and points to the need for future studies to look in more detail at the mechanisms and regulatory dynamics TH membrane transport in teleost fish models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TH	thyroid hormone
T_3	3,5,3′-triiodothyronine
T_4	thyroxine
Mct	monocarboxylate transferase
Oatp	organic anion-transporting polypeptide
Slc	solute carrier protein

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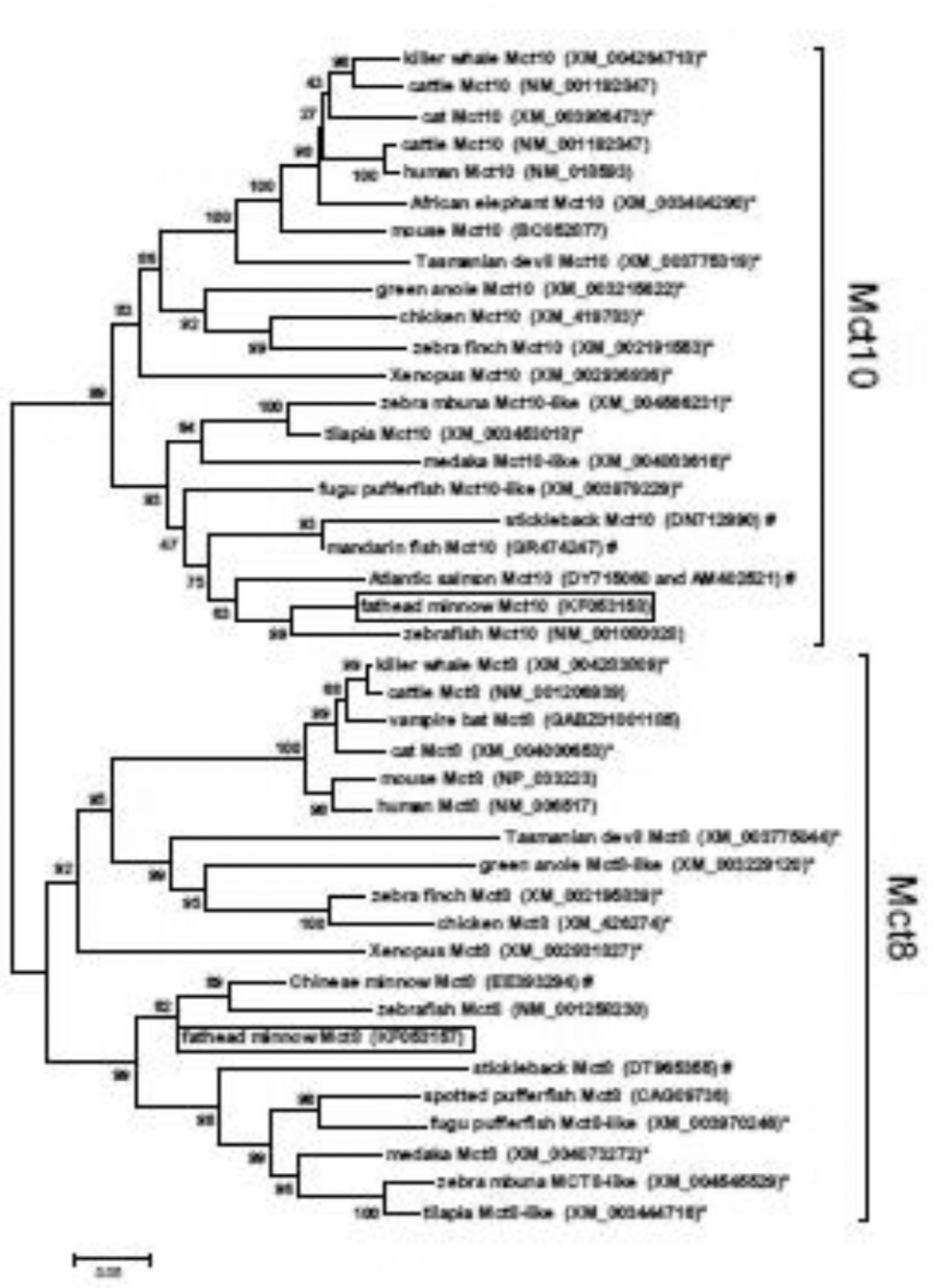

Figure 1.

Phylogenetic tree based on alignment of deduced amino acid sequenced for Mct8 and Mct10 from teleost fishes and other select vertebrates. Tree was assembled using the neighbor-joining method with pairwise deletion, and bootstrap values (1000 replicates) are indicated at each node. Boxes indicate the *mct8* and *mct10* cDNAs identified from fathead minnow; (*) indicates predicted proteins based on sequenced genomes; (#) indicates partial proteins deduced from unannotated EST sequence. GenBank accession nos. are shown following each taxon designation.

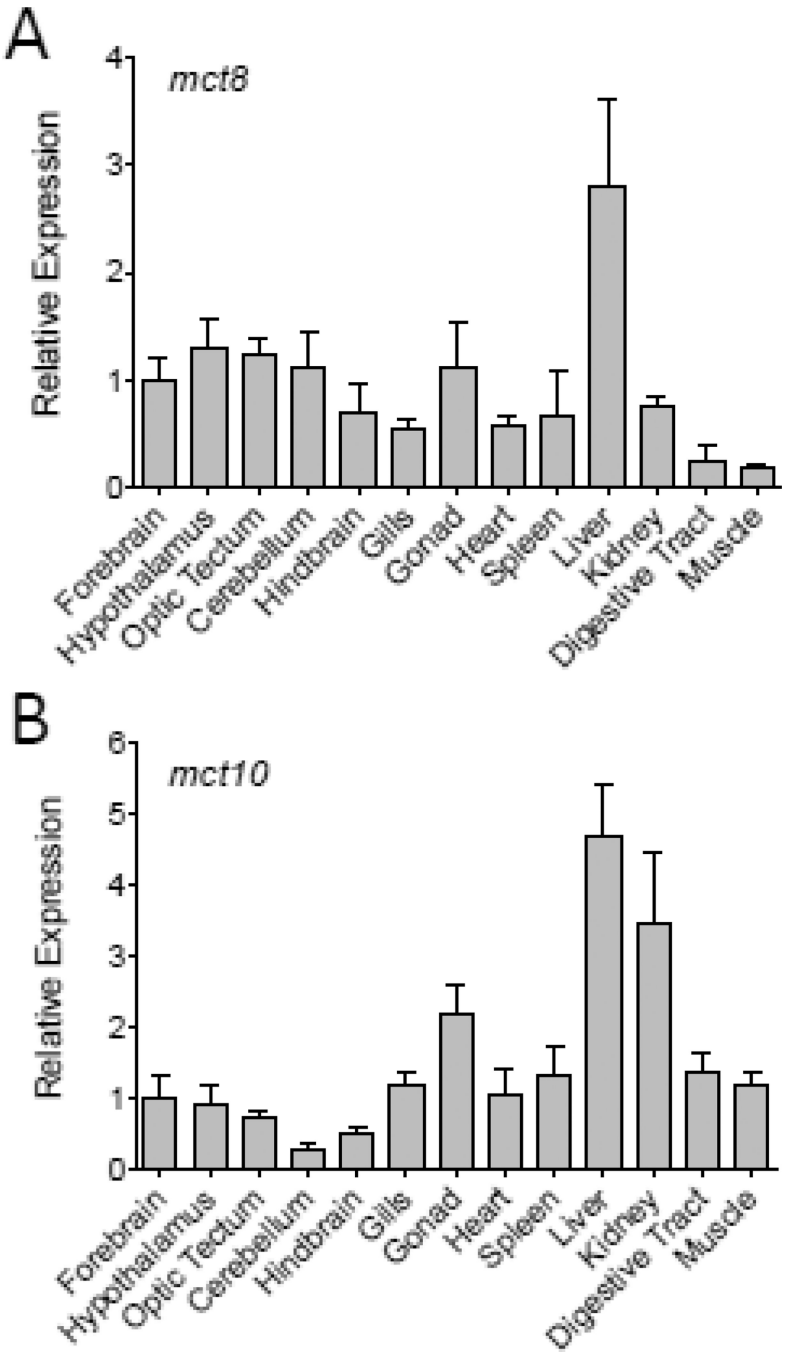

Figure 2.

Tissue distribution of *mct8* (**A**) and *mct10* (**B**) mRNAs in adult male and female fathead minnows (n = 6). Data values are shown as mean ± SEM. Relative expression values for each transcript are normalized against the mean abundance value of the forebrain tissue.

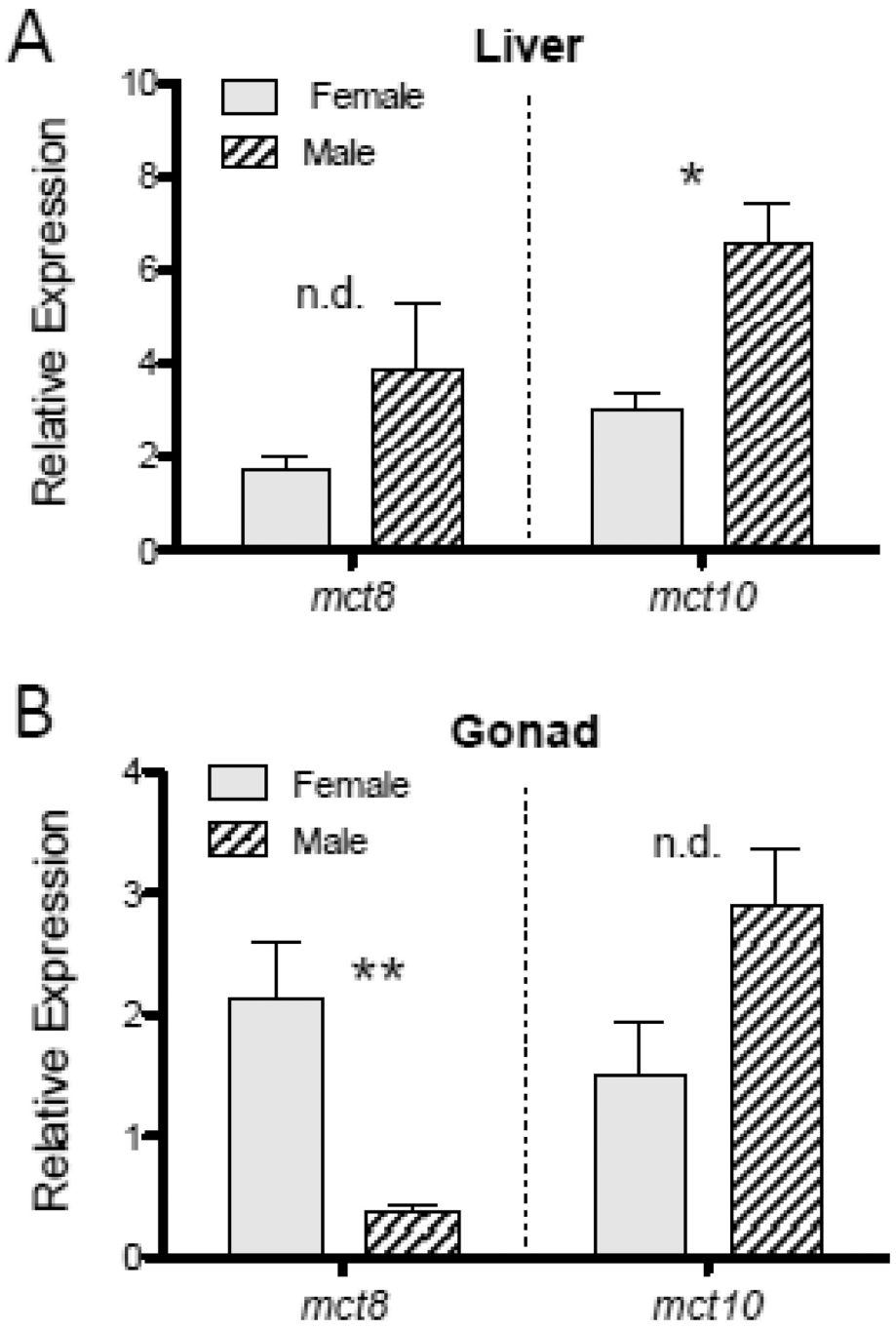

Figure 3.

Sex differences in the relative abundance of *mct8* and *mct10* mRNAs observed in the liver (**A**) and gonad (**B**) tissues of adult fathead minnows. Data are shown as mean ± SEM, with * indicating $p < 0.025$, ** indicating $p < 0.01$, and *n.d.* indicating 'no difference' between sexes. Data values are normalized to the mean abundance value for that same gene transcript in the forebrain. Sample sizes are $n = 3$ fish per gender.

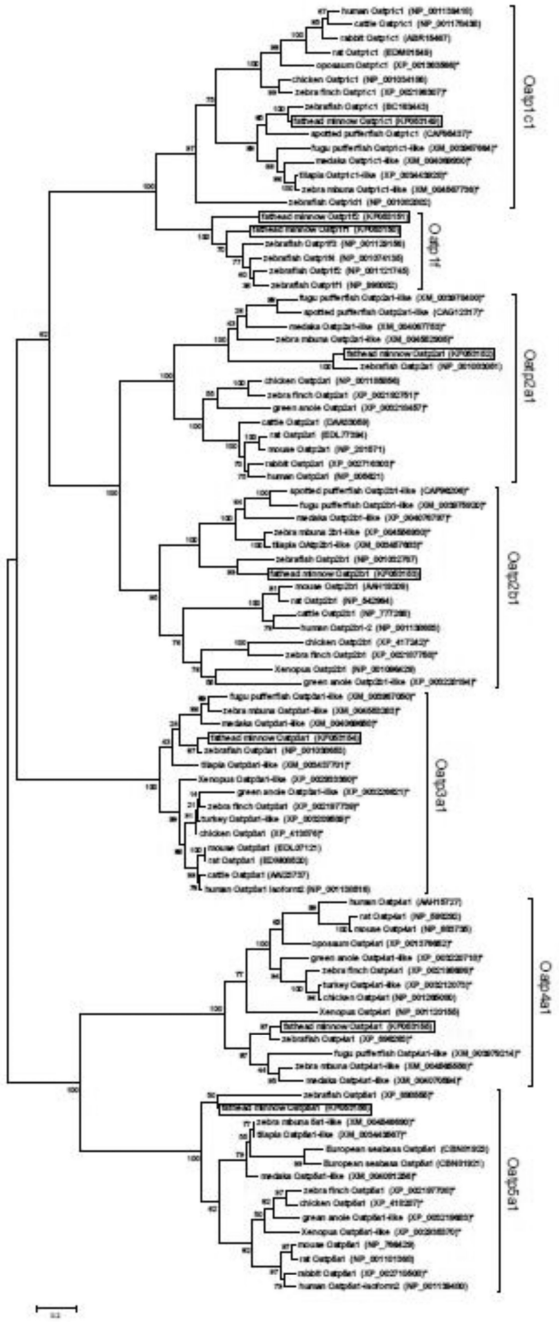

Figure 4.

Phylogenetic tree from alignment of deduced amino acid sequences for Oatps from fish and select vertebrates. Tree was assembled by maximum likelihood using a Jones-Taylor-Thornton model (Jones et al., 1992) with all sites. Bootstrap values (1000 replicates) are indicated at each node. Boxes denote the eight Oatp cDNAs identified from fathead minnow. Asterisks (*) indicate predicted sequences based on genome projects, and GenBank accession nos. are provided following each taxon name.

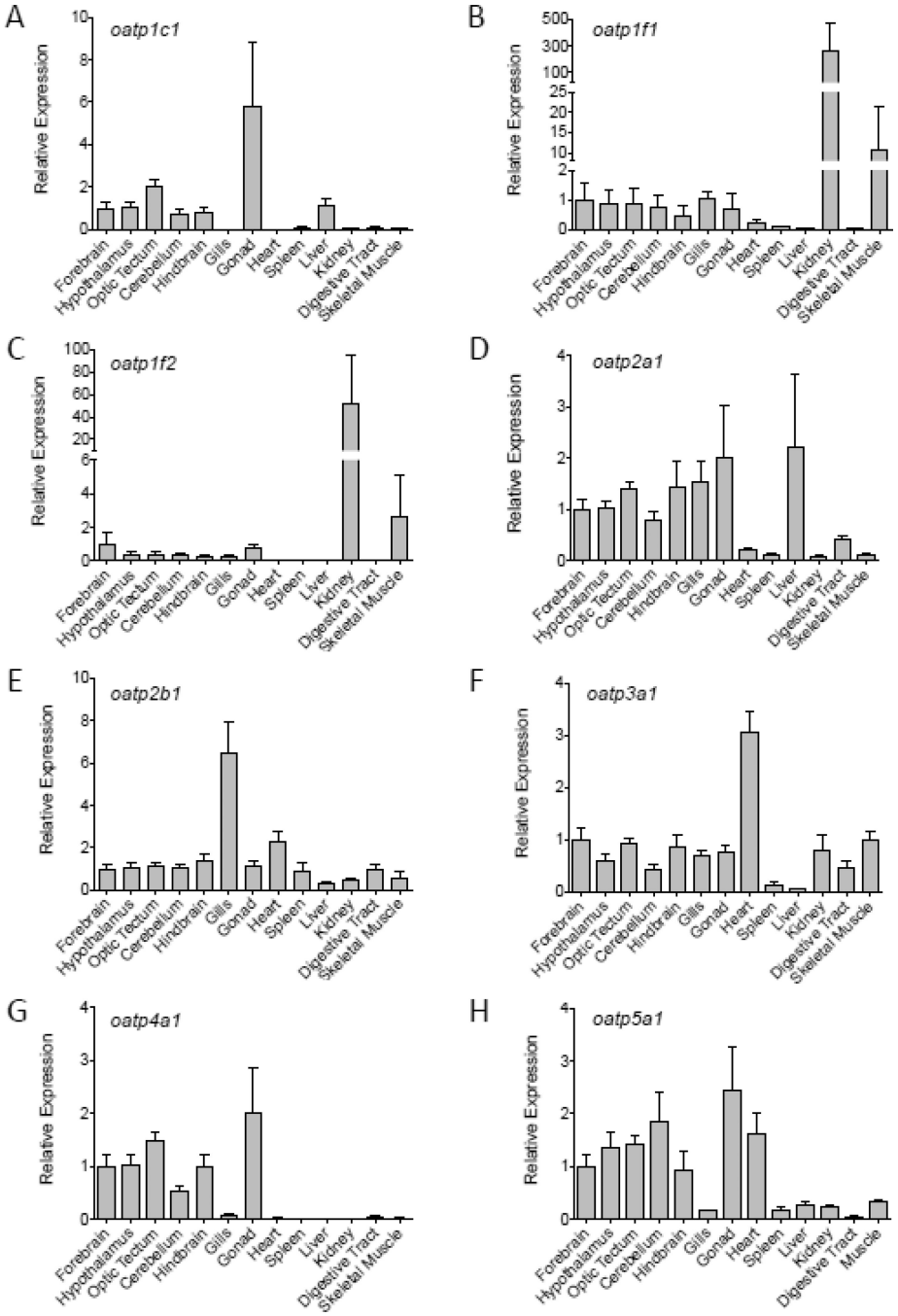

Figure 5.

Relative *oatp* mRNA levels among tissues of adult fathead minnows (n = 6). Data values are shown as mean ± SEM. Relative expression values for each transcript are normalized against the mean relative abundance value for that same transcript in forebrain tissue.

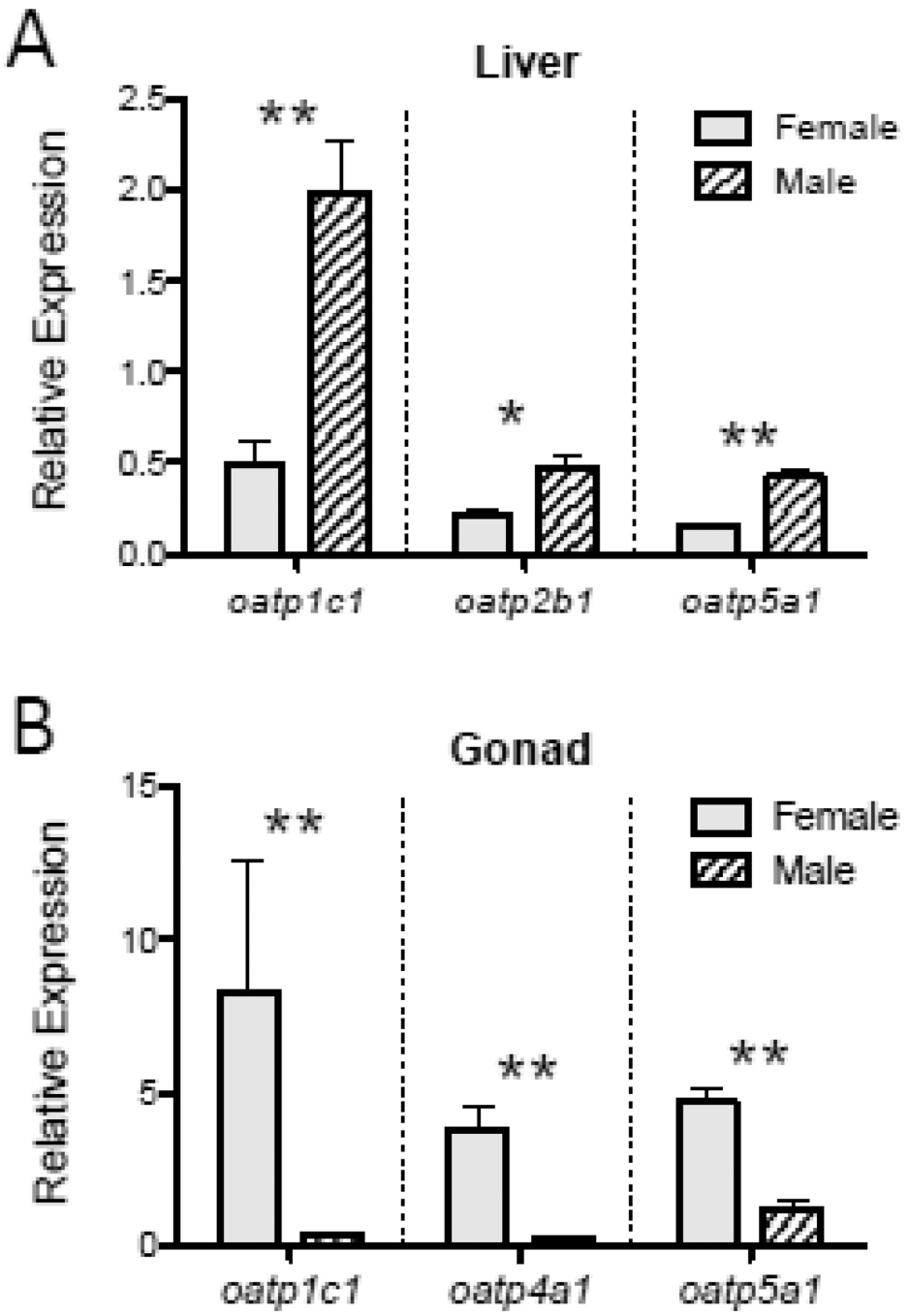

Figure 6.

Sex differences in *oatp* mRNA transcript abundance in the liver (**A**) and gonadal (**B**) tissues of adult fish. Data are shown as mean ± SEM, with * indicating $p < 0.025$, and ** indicating $p < 0.01$. Data values are normalized to the mean abundance value for that same gene transcript in the forebrain (see Fig. 5). Sample sizes are $n = 3$ fish per gender.

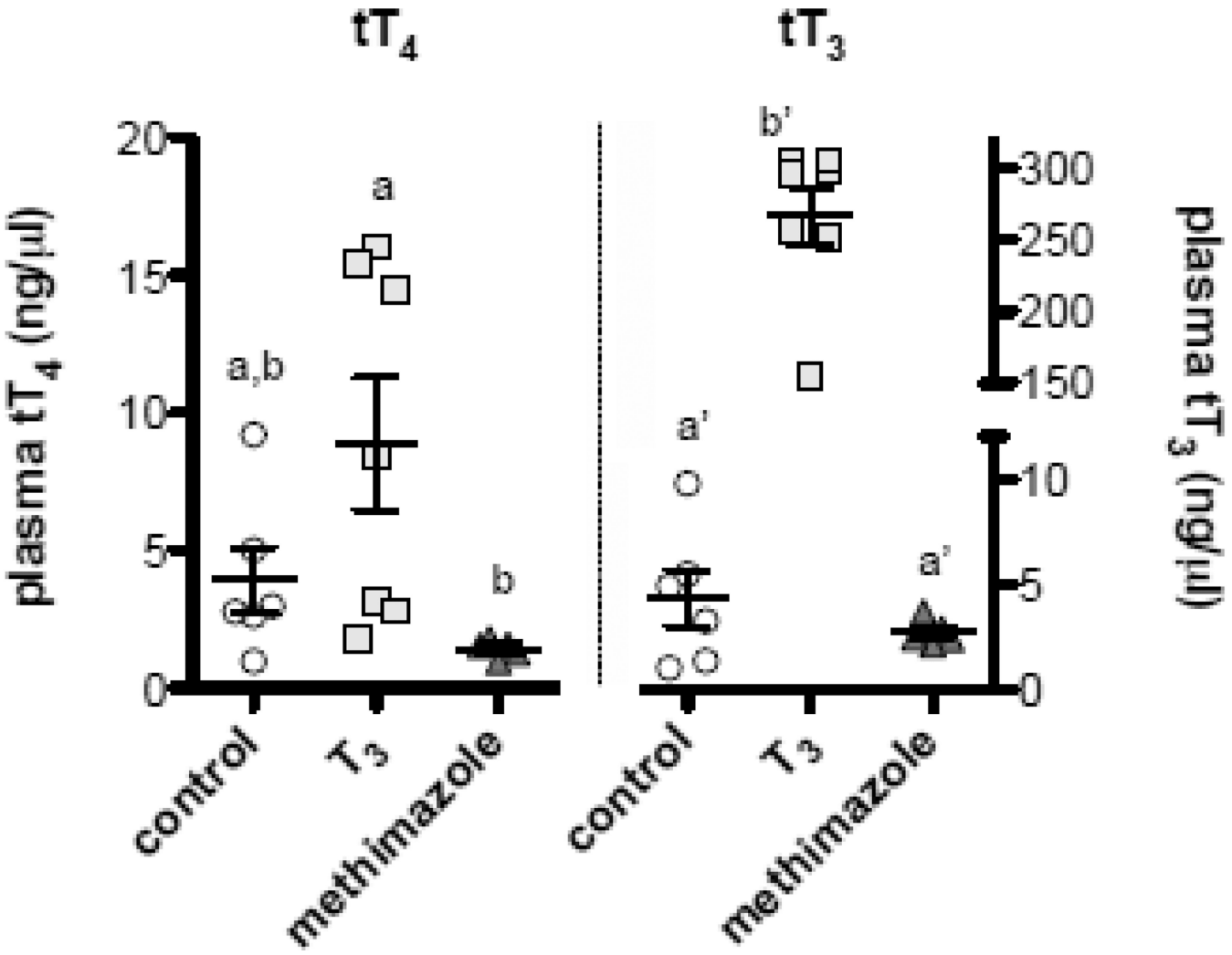

Figure 7.

Plasma total T_4 (tT_4) and total T_3 (tT_3) concentrations in adult male minnows given dietary treatments of control vehicle, exogenous T_3, or the goitrogen methimazole for 14 days. Methimazole depressed tT_4, while exogenous T_3 significantly elevated plasma tT_3 concentrations over control and methimazole-treated fish. Each data point represents the hormone concentration measured from the pooled plasma of n = 3–6 minnows, and bars denote mean ± SEM values for each treatment group. Letters indicate pairwise differences among treatment groups for each hormone (Tukey HSD tests, $\alpha = 0.05$)

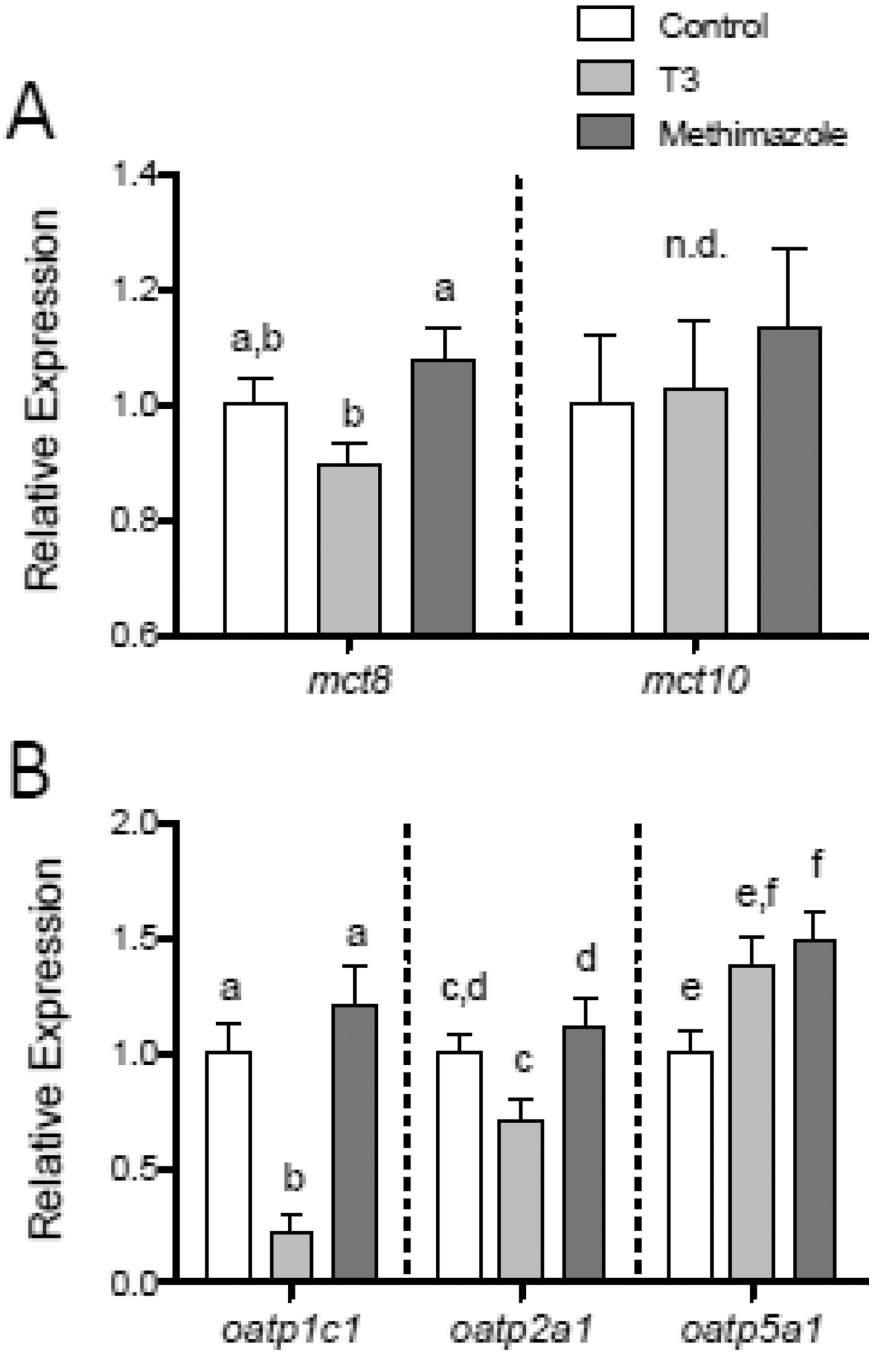

Figure 8.

Variation in relative levels of *mct* and *oatp* transcripts in the liver with experimentally-induced changes in circulating TH concentrations. Transcripts encoding *mct8*, but not *mct10*, were reduced in male fathead minnows treated with exogenous T_3 for 14 days, compared to fish treated with the goitrogen methimazole. (**A**). Transcripts encoding *oatp1c1* and *oatp2a1* were likewise down-regulated in the liver of T_3-treated male fish, while *oatp5a1* mRNAs became elevated in the liver of both T_3- and methimazole-treated (**B**) in the liver. Letters indicate significant differences between groups for each transcript (Tukey

HSD tests, $p < 0.05$), and *n.d.* indicates “no difference” among treatment groups. Sample sizes are $n = 16–18$ fish per treatment.

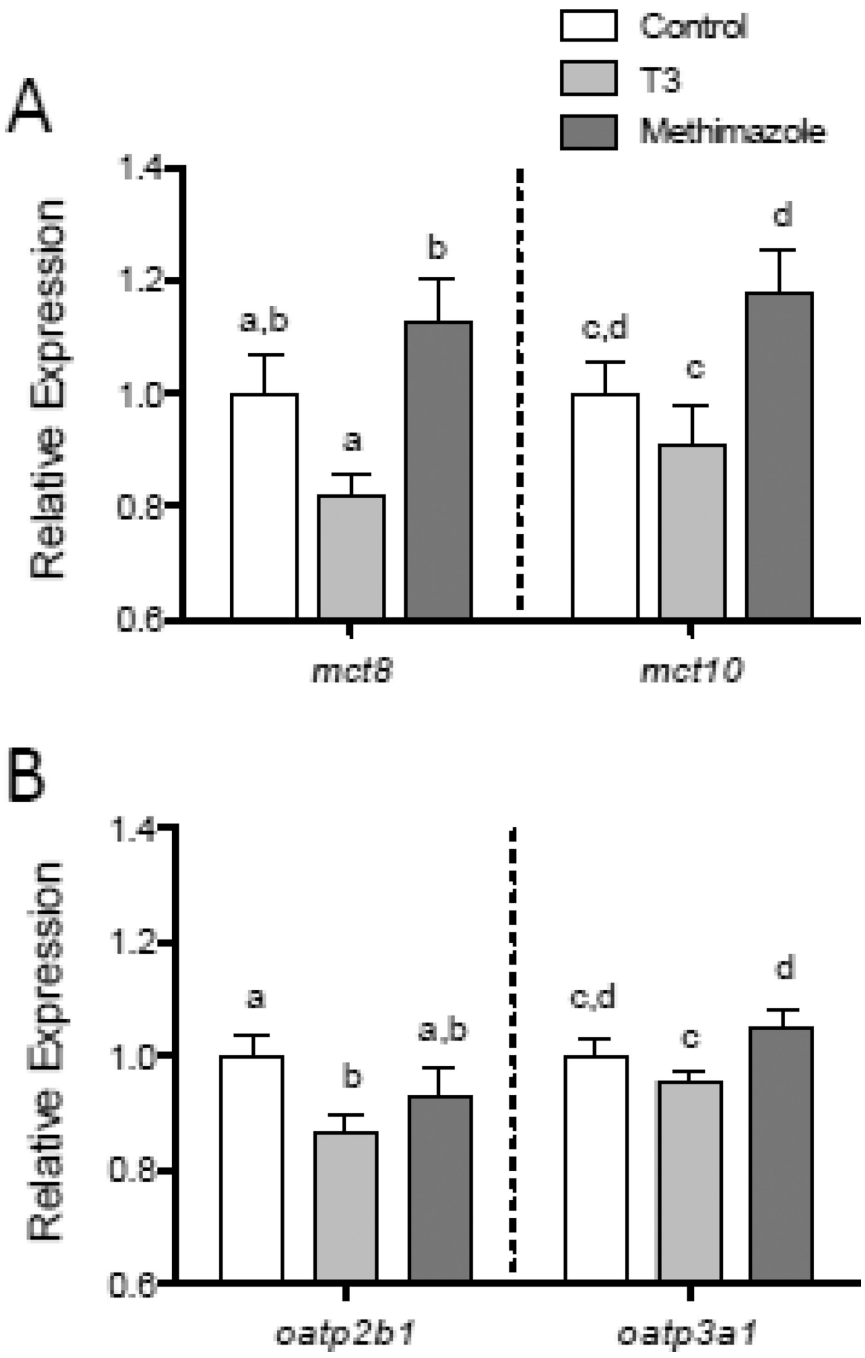

Figure 9.

Changes in *mct* and *oatp* relative mRNA levels in the brain of male fish treated with exogenous T_3 or methamizole for 14 days. Transcripts encoding *mct8* and *mct10* were both found to be lower in the brain of T_3-treated fish compared to methimazole-treated fish (**A**). Transcripts encoding *oatp2b1* and *oatp3a1* also showed reduced relative mRNA levels in the brain of T_3-treated fish (**B**). Letters indicate significant differences between groups for each transcript (Tukey HSD tests, $p < 0.05$). Sample sizes are $n = 16–18$ fish per treatment.

Table 1

Nucleotide sequences for primer and Taqman probe used in quantitative real-time RT-PCR.

Transcript	Primer	Nucleotide Sequence (5′ to 3′)	Amplicon Size (bp)	% Efficiency (Avg.)
mct8	Forward primer	ACCAGGCGTCTCAGTTTAAAGTG	72 bp	97.55%
	Probe	CATGGGTCGGCGCTCTGGC		
	Reverse primer	CCGGCGAGCAGAAGAAGAT		
mct10	Forward primer	GCTCTCCGATCGTCAGTGTGT	61 bp	94.96%
	Probe	CACGGATCTGCTGGGATGCCG		
	Reverse primer	TCCTCCGACAGCCGTGAT		
oatp1c1	Forward primer	GAGAGACGGTCACCGGAGAA	67 bp	93.70%
	Probe	TCCAAGGCCCTGTTGCTCAAGCC		
	Reverse primer	AGGCCACGAGGAACATCTTG		
oatp1f1	Forward primer	TGACGCCCAGAATACTGTTCAA	64 bp	98.17%
	Probe	AGCTTGTGGCCGGATGCCA		
	Reverse primer	CGAGCTTTCCTCTTCCCCTTT		
oatp1f2	Forward primer	TGCAGAATGTATGACACTGAGACTT	71 bp	95.03%
	Probe	AGGTTCCTGTTTCTGGGACTGG		
	Reverse primer	CCTAACACCCGCAGAGAGTTG		
oatp2a1	Forward primer	TGGCCTTGACAGCCTCTCTT	65 bp	93.43%
	Probe	AGGAACCGTCTCGTCCCTCCAT		
	Reverse primer	TCAGCACTGTGTTCCCAACCT		
oatp2b1	Forward primer	GAAAAATGGTGACGCCAGATG	65 bp	96.94%
	Probe	TTGGAGCTTGGTGGTTGGGA		
	Reverse primer	GGAGGGTGGCAGCTATCAAG		
oatp3a1	Forward primer	CAACCTGGCCCTCATTCTCTTTGT	168 bp	98.34%
	Probe	ATGGCTTTGGGAGCGCTGCTCTCCGCATTA		
	Reverse primer	CCCAATTCCTTGCTCCATGTGTCT		
oatp4a1	Forward primer	TCGCTCATTGCAGGCATCATCTAC	160 bp	94.34%
	Probe	AGCTGTGAGAGCACAGATGTGGGAGGCCAT		
	Reverse primer	TGATTGGCAGGTCTCTGTTCTTCC		
oatp5a1	Forward primer	TTGACCAGAGTGACCCACGATTTG	169 bp	92.46%
	Probe	TGGAGTGGGTTTCTCCTGTGTGCTGTGGCA		
	Reverse primer	CTGGACACATCACCTGCACTCTTT		
ef1α	Forward primer	GTTTGAGAAGGAAGCTGCCGAGAT	100 bp	98.50%
	Probe	AGGGCTCCTTCAAATATGCCTGGGTGCT		
	Reverse primer	ATCAATGGTGATACCACGCTCAC		

Transcript	Primer	Nucleotide Sequence (5′ to 3′)	Amplicon Size (bp)	% Efficiency (Avg.)
rpl8	Forward primer	CTGTTGTTGGTGTTGTTGCTGGTG	95 bp	94.92%
	Probe	ACCCATCCTGAAGGCAGGACGTGCATACCA		
	Reverse primer	GCAGTTCCTCTTGGCCTTGTACTT		