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Characterization of thyroid hormone transporter expression during tissue-specific metamorphic events in *Xenopus tropicalis*

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ABSTRACT

Thyroid hormone (TH) induces the dramatic morphological and physiological changes that together comprise amphibian metamorphosis. TH-responsive tissues vary widely with developmental timing of TH-induced changes. How larval tadpole tissues are able to employ distinct metamorphic programs in a developmental stage- and TH-dependent manner is still unknown. Recently, several proteins capable of transporting TH have been identified. TH action and metabolism occurs primarily intracellularly, highlighting the importance of TH transporters. We examined the hypothesis that TH transporter expression and tissue distribution play an important role in mediating TH-induced metamorphic events. *Xenopus tropicalis* homologs for known TH transporting OATP, MCT and LAT family proteins were identified and gene specific qRT-PCR primers were developed. Total RNA was extracted from tissues representing three unique developmental fates including: growth/differentiation (hind limb), death/resorption (gill, tail) and remodeling (brain, liver, kidney). For growing and resorbing tissues, results showed the general trend of low initial expression levels of MCT8 and MCT10 transporters, followed by a several-fold increase of expression as the tissue undergoes TH-dependent metamorphic changes. The expression pattern in remodeling tissues was less uniform: a general decrease in transporter expression was observed in the liver, while the kidney and brain exhibited a range of expression patterns for several TH transporters. Collectively, these developmental expression patterns are consistent with TH transporting proteins playing a role in the effects of TH in peripheral tissues.

1. Introduction

Amphibian metamorphosis is a thyroid hormone (TH; 3,3′, 5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4)) dependent process that systematically transforms most, if not all, larval anuran tissue in profound morphological or physiological ways (for review see Dodd (1976) and Shi (2000)). Metamorphic changes are described as occurring in three distinct phases, relative to circulating TH concentrations: premetamorphosis with an absence of TH, prometamorphosis (stages 55–57 by Nieuwkoop and Faber, 1994; (NF 55–57)) characterized by rising TH concentrations, and metamorphic climax (NF 58–65) where TH levels are at their maximum Dodd (1976). Some tissues, like the hind limb, exhibit TH-induced growth and development early in prometamorphosis (NF 53–54) when plasma concentrations of TH are minimal (Leloup and Buscaglia, 1977; Nieuwkoop and Faber, 1994). Other metamorphic programs, such as gill and tail resorption and intestinal remodeling, occur in rapid succession during metamorphic climax (Nieuwkoop and Faber, 1994). How larval tadpole tissues are able to employ distinct metamorphic programs in a developmental stage- and TH-dependent manner is still unknown.

The thyroid gland primarily produces a prohormone T_4, which must be converted into the biologically active T_3 through the action of deiodinase selenoproteins before it can exert genomic level effects. Two classes of iodothyronine deiodinases have been characterized in *Rana catesbiana* tadpoles (Becker et al., 1997). Type II deiodinases (DII) catalyze the deiodination of the outer ring of a thyroid hormone. This protein is often referred to as the 'activating' deiodinase, as it converts T_4 into the biologically active T_3. Alternatively, type III deiodinase (DIII) catalyzes inner-ring deiodination, resulting in the production of reverse T_3 (rT_3), a biologically inactive hormone derivative. It has been hypothesized that the distribution of deiodinase expression may explain the coordination of metamorphosis in TH-sensitive tissues by inhibiting or promoting the presence of the active T_3 hormone. Supporting this hypothesis, increasing DII activity and mRNA expression has been shown to correlate with the timing of TH-sensitive metamorphic changes in hind limbs, fore limbs, tail, and the intestines (Becker et al.,

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1997; Cai and Brown, 2004). If DIII is over expressed, however, a retardation or complete arrest of metamorphosis at NF stages 60–61 is observed (Huang et al., 1999). While this hypothesis may help explain the metamorphic patterns of some tissues, DII has not been observed in the larval amphibian liver (Becker et al., 1997), kidney (Becker et al., 1997) and gill tissues (Cai and Brown, 2004). Differential expression of thyroid hormone nuclear receptor (TR) proteins has also been examined as an alternative explanation for the temporal and tissue-specific effects of TH during metamorphosis (Kawahara et al., 1991; Wong and Shi, 1995; Yaoita and Brown, 1990). The role of TRs in facilitating tissue-specific metamorphosis is complicated however by the TH-induced up-regulation of these proteins (Kanamori and Brown, 1992; Yaoita and Brown, 1990).

TH action and metabolism requires that the hormone is transported from the bloodstream across the plasma membrane and into a cell in order to exert its gene transcription effects (Hennemann et al., 2001). Transport of TH across the plasma membrane has been shown to be a rate-limiting step in TH conversion (Hennemann et al., 1986) and, presumably, in the activation of thyroid hormone gene transcription (Ritchie et al., 2003). A variety of proteins capable of TH transport have recently been described, including members of the organic anion transporting polypeptide (OATP) (Fujiwara et al., 2001; Hagenbuch and Meier, 2003), L-type amino acid transporter (LAT) (Friesema et al., 2001) and monocarboxylate transporter (MCT) families (Friesema et al., 2003, 2008). The majority of TH transporters currently known have low specificity for TH, and are capable of transporting a wide range of substrates. However, OATP1C1 (Pizzagalli et al., 2002), MCT8 (Friesema et al., 2003) and MCT10 (Friesema et al., 2008) are capable of high-affinity, high-specificity TH transport. In this study, we examined the hypothesis that TH transporter expression and tissue distribution may play an important role in mediating TH-induced metamorphic events.

2. Materials and methods

2.1. Animal care and culture

Xenopus tropicalis "golden strain" were originally obtained from the University of California, Berkeley lab of Richard Harland. The breeding pair used to generate animals for this study was the product of at least three generations of inbreeding. Breeding was induced by an injection of human chorionic gonadotropin (Sigma Aldrich, St. Louis, MO). Both the male and female were injected with 20 IU of the hormone followed 5 h later by a second injection of 100 IU. The resulting tadpoles were maintained in 25 °C ozone treated Lake Superior water in 7-l aquarium tanks at a density of 30 tadpoles per tank. The tanks received continuous inflow of water at a rate of 50 ± 2 mL/min. Tadpoles were fed a mixture of brine shrimp, spinach and Sera Micron (Sera North America, Toronto, Canada).

2.2. Experimental design

Tadpoles were anesthetized in 100 mg/L of MS-222 buffered with 200 mg/L of sodium bicarbonate and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). For each time point highlighted in the dissection outline below (Table 1), tissues from five individual tadpoles were dissected and immediately preserved in RNAlater™ (Ambion, Austin, TX) with the exception of the brain tissues, which were immediately homogenized in RLT buffer with an electric pestle. Sampling time points for each tissue were chosen to capture periods before, during, and after a TH-mediated change was expected. Whole tails, gills, kidneys and hind limbs were collected. The pituitary and hypothalamus were dissected out of the brain for individual analysis. Liver samples consisted of a single lobe.

Table 1
Dissection outline. Shaded boxes indicate assayed developmental stages.

Tissues	Developmental stage (NF)											
	53	54	55	56	57	58	59	60	61	62	63	64
Hind limb	■	■	■	■	■	■						
Liver				■	■	■	■	■	■	■	■	■
Tail				■	■	■	■	■	■	■	■	■
Gill				■	■	■	■	■	■			
Kidney		■		■		■		■		■	■	■
Brain		■		■		■		■		■		■

2.3. RNA extraction

Total RNA was isolated from tail, gill and hind limb samples using TRI REAGENT™ according to manufacturer's instructions (Sigma–Aldrich, St. Louis, MO, USA). Samples were mechanically disrupted in 600 μL TRI REAGENT™ in a 2.0 mL safe-lock Eppendorf microcentrifuge tube with a 5 mm stainless steel bead (Qiagen, Valencia, CA). Samples were homogenized in a Retsch MM301 Mixer Mill (Retsch Inc., Newtown, PA, USA) at 30 Hz for 7 min. Mixing chambers were rotated 180° halfway through the homogenization procedure. After extraction, RNA pellets were reconstituted in nuclease-free water. Total RNA from liver and kidney samples were extracted according to the manufacturer's instructions using the RNeasy Plus Mini and Micro kits, respectively (Qiagen, Valencia, CA). Samples were homogenized as described above in 600 μL of RLT Plus buffer with 0.5% Dx reagent (Qiagen, Valencia, CA). Brain, pituitary and hypothalamus samples were extracted according to the manufacturer's instructions using RNeasy Micro kits (Qiagen, Valencia, CA).

RNA quantity and purity was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). For quality control purposes, two of the five samples for each tissue at each stage were assayed with an RNA Pico LabChip kit on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). For all tissues RIN (RNA Integrity Number) values ranged between 9 and 10. This is indicative of RNA with high quality that is acceptable for downstream applications. These samples were diluted to the following final concentrations: tail and gill, 6 ng/μL; liver, 5 ng/μL; brain, 4 ng/μL; hind limb and kidney, 3 ng/μL; and stored at −80 °C until subsequent use. Due to low RNA quantity and quality of pituitary and hypothalamus samples, these tissues were omitted from further analysis.

2.4. Locating X. tropicalis TH transporter orthologs

A literature search was performed to identify known thyroid hormone transporting proteins. *X. tropicalis* transporter orthologs obtained from the Xenbase v.2.4 database (www.xenbase.com) include MCT8, MCT10, LAT1, OATP1C1, OATP4C1, OATP1B3 and OATP4A1 (Transcript Accession Numbers: e_gw1.10.317.1; C_scaffold_250000006; e_gw1.188.92.1; e_gw1.1610.1.1; e_gw1.58.3.1; e_gw1.1610.7.1; e_gw1.1016.4.1, respectively). A putative MCT7 gene ortholog was identified using *X. laevis* protein sequence (Accession: AAH47967.1) as a query to search the *X. tropicalis* genome build 4.1 (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). Correct annotation of this sequence was verified by blasting the resulting *X. tropicalis* sequence in the NCBI database (National Center for Biotechnology Information; NCBI). All top query results yielded identically annotated proteins, thus validating this sequence as a *X. tropicalis* MCT7 ortholog. Additional TH transporters of interest that

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were not successfully located include OATP6B1, OATP6C1, OATP1A1, OATP1A2, OATP1A4, OATP1A5, OAPT1B1 and OATP1B2.

2.5. Real-time quantitative polymerase chain reaction (qRT-PCR)

Gene-specific nested primers were designed with the aid of PrimerExpress (Applied Biosystems) and were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primers were designed to encompass regions indicative and unique to each transporter (Table 2). Additionally, qRT-PCR primers overlay an exon–exon junction.

QPCR standards were generated by performing PCR using outer primers with an Enhanced Avian HS RT-PCR kit (Sigma, St. Louis, MO) (Table 3). Products were run on an agrose gel to verify that a single product of correct amplicon size was produced. PCR product was then reamplified using middle primers to incorporate the T7 promoter region (Jumpstart Taq, Sigma, St. Louis, MO). DNA sequencing verified the product's identity (Biotechnology Resource Center, Cornell, NY). Standard RNA was generated from middle PCR products using a MEGAscript kit (Ambion, Austin, TX). Standard purity, concentration and size were verified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and an RNA Pico LabChip kit on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

Quantitative real-time PCR assays were performed using a Taqman® EZ RT-PCR kit (Applied Biosystems) according to the manufacturer's protocol. Samples were reverse transcribed (50 °C for 2 min, 60 °C for 30 min, 95 °C for 5 min) and then subjected to 40 cycles of PCR amplification (melt 94 °C for 20 s, anneal and extend 58 °C for 60 s) using a 7500 Real-Time PCR machine (Applied Biosystems). A standard curve of known concentration (copies/μL) was used to quantify qRT-PCR sample data. Standard curve values ranged in concentration from 10^2 to 10^8 copies per sample, and were created in serial dilutions. Using the standard curve, samples were converted to copies of gene/ng total RNA for each sample.

Table 2
Real-time PCR primer and probe sequences for TH transporting proteins.

Gene of interest	Real-time PCR primer and probe sequences (5′ → 3′)
OATP4A1	F: CCATTAAGGACCTGCCAAAGTC R: CCCCGCTAAGCACAGGAA P: TTGATGCTTCTCAAGAACCCGACGTTTG
OATP1C1	F: TGGGAGCGTCTTCCAGTACAA R: GTCCGTACTGCTGCTCGATGT P: CCCTGATCGGCATGGTCACCTACAAA
OATP4C1	F: GATCCATTCGCAGGTGTATCTG R: CTCCATCCGATCCACAAACTG P: ATGGGACTGGAAAGATTGGAGAATTAGATGCC
MCT8	F: ATTGGTTACGTTCTTGGACTCATG R: ACGTAAGAGGCCTGCAATGG P: CCATCCCAATGACTGCTGGACCG
MCT10	F: TTGGTCCACCTATTGCAGGTATT R: TGGATCCATGGTATCAGGCATA P: CCGCCTTGGTTCTTATGATGTTGCATTC
LAT1	F: GGCCTATCAAGGTAAACATTTTGC R: CACACTCAACAGGTGTCATGTAGAAT P: TTTTTTTCATCCTTGCCTGCTTTTTCCTCA
OATP1B3	F: AAGACGTTGTGTTTCAACCGATT R: GAAGCCAAGGAAGCTGTTGGT P: TTGTCATGTTGATGTGCCTCACCCTCC
MCT7	F: TGCTTCGCCGTCTTTGC R: CCTCCAGCCAATCAGATCGT P: TTTGCCCCAGCCATCACTGCAC

F = forward primer. R = reverse primer.
P = dual-labeled probe with 5′-FAM, 3′-Black Hole Quencher 1.

Table 3
Gene-specific primers used to create RNA standards for qRT-PCR. The product from outer primers (Out) was amplified with middle primers (Mid) that contain a promoter for T7. Promoter is depicted in lower case.

Gene	Primer (5′ → 3′)	Amplicon (bp)
MCT7	Mid F: taatacgactcactatagggagg GCCGCTCGCTGGTTACC Mid R: TTTCCAGCGCTTCACCTTTG Out F: CGATCCTGTCGCAGTATTTCG Out R: TGAGCGTCTGCTCGTTCTCA	248 285
MCT8	Mid F: taatacgactcactatagga TGAGTTAGTGGGACCCATGCA Mid R: GATGCCAGCCAGATAGAATGC Out F: CTAATCAGTATGATGAGCCCTATTGC Out R: CCAGGCAGAAGCTCTCCATTC	152 311
MCT10	Mid F: taatacgactcactatagggagg ATTTTTGCTTGGCTTTATGTCCAT Mid R: CTATTGTTTTCTTCTGCAAAGTCCTTT Out F: GGCTCCAATTGCCTTTGAAC Out R: CTTCAGCATCTCCATTTACAGCTATT	185 260
LAT1	Mid F: taatacgactcactatagggagg CAGCTTCTTTAACTGGCTTTGTGTAG Mid R: ACAGGAACACCAGACAAAATAATGAT Out F: TGCGCCATGACACTCCTCTA Out R: TGCCATGCAGGAGCCATT	213 325
OATP1C1	Mid F: taatacgactcactatagggagg CGTCGGTGAAGGATCTTTTTG Mid R: GGAAGTTTGTCTTGGAGGAGGTT Out F: AGGCAACTACGGAGCAAGATG Out R: CCCCCCGGAGAAAATGC	150 260
OATP4C1	Mid F: taatacgactcactatagggagg TGGCATCCGTTTTTATTTTTGC Mid R: CAGACGTACACCCAGCATAGCA Out F: GGCGTAATTGTCTCCAAATGTAAAT Out R: CCGTATATCTTTCCTTTATCATTGTATTCC	201 314
OATP1B3	Mid F: taatacgactcactatagggagg GGGCAGAAACCCCAGACTAAG Mid R: GCCATACTGCTGCTCCATGTAC Out F: TCCTACCCGAGAGCCTGAAAG Out R: CTTGGAGATGGACTGGCCATA	225 275
OATP4A1	Mid F: taatacgactcactatagggagg CTGAAGCGCATCAGGTGAAA Mid R: AAGTGGCAGCTTGTGATGCA Out F: AGCCTACCCCCAGCAGCTA Out R: GGAACCACCAAATATCCAAAGC	225 300

If contamination was detected in NTC (no template control) or if the 10^2 standard exhibited inefficient amplification, the 10^2 standard was omitted from the standard curve. This resulted in varying limits of detection (Table 4). All samples, including a NTC, were run in duplicate in order to estimate intra-assay variability. Intra-assay coefficients of variation (CVs) were calculated for each sample. Samples containing CV's of >15% were discarded.

2.6. Statistical analysis

Data from all tissue/gene combinations were assessed for normality and equal variance using Kolmogorov–Smirnov and Levene Median tests, respectively. A one-way analysis of variance (ANOVA) was used to test for differences between developmental stages when data met parametric assumptions. Multiple pair-wise comparisons were then performed using the Student–Newman–Keuls post hoc test. When data did not conform to parametric assumptions, a nonparametric Kruskal–Wallis one-way analysis of variance on ranks was performed with a Student–Newman–Keuls post hoc test or a Dunn's post hoc if group sizes were unequal. Differences in expression levels were considered significant at $p < 0.05$. All statistical analyses were conducted using Sigmaplot 11.0 (Systat Software, Inc., Chicago, IL).

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Table 4
qRT-PCR limit of detection in copies/ng total RNA.

	OATP1C1	OATP4C1	OATP1B3	OATP4A1	MCT7	MCT8	MCT10	LAT1
Gill	3.3	33	33	3.3	3.3	3.3	33	3.3
Tail	3.3	33	33	3.3	3.3	3.3	33	3.3
Limb	5	50	50	50	5	5	5	5
Kidney	6.7	67	6.7	6.7	6.7	6.7	6.7	67
Liver	4	40	4	4	4	4	4	40
Brain	5	50	5	5	5	5	5	5

3. Results

To examine potential gene expression patterns during metamorphosis, the expression of MCT7, MCT8, MCT10, LAT1, OATP1C1, OATP4C1, OATP1B3 and OATP4A1 were analyzed using qRT-PCR. Several unique transporter expression patterns were observed in each tissue.

3.1. Hind limb

Most gene expression patterns in the developing hind limb were associated with an increase in expression from NF stages 5 to 54. Transporters MCT10, LAT1 and OATP1C1 (Fig. 1c–e) exhibited a significant increase in expression at NF stage 54, followed by relatively stable expression. MCT7 followed this trend, but continued to gradually increase in expression levels during all stages examined (Fig. 1a). The large standard deviation observed during NF stage 54 was caused by two samples detected in levels of 800 copies/ng total RNA, concentrations four times higher than the other three samples measured at this stage. Another transporter, OATP1B3 (Fig. 1g), exhibited the same increase in expression at NF stage 54 but immediately return to a low level of expression during the remaining metamorphic stages. A similar pattern was observed in the genes MCT8 and OATP4C1 (Fig. 1b and f), but these genes gradually returned to basal expression levels by NF stage 57. The exception to this expression pattern is OATP4A1, which was expressed at levels at or below the detection limit until NF stage 57 and then stably expressed at 97.8 ± 20.5 copies/ng total RNA (data not shown).

3.2. Kidney

The remodeling kidney exhibited a variety of gene expression patterns. Two transporters, MCT8 and MCT10 (Fig. 2c and d), showed a significant up-regulation in expression over developmental time. This increase was more gradual and less pronounced in MCT10. The transporter LAT1 also showed a pattern of a gradual increase in expression, peaking at NF stage 62/63 (Fig. 2d). In contrast, two genes showed constant levels of expression with a spiking 2-fold increase during a single NF stage: NF stage 58 for MCT7 and NF stage 60 for OATP4C1 (Fig. 2a and e). No significant expression pattern was observed for OATP4A1; values ranged from 12.3 ± 3.3 to 47.8 ± 67.9 copies/ng total RNA (data not shown). Further, transporters OATP1C1 and OATP1B3 were expressed at or below the limit of detection (data not shown).

3.3. Liver

Most transporters observed in the remodeling liver followed a pattern of decreasing gene expression. Transporters MCT7 and OATP1B3 both significantly decreased (NF stages 59 and 57, respectively) to stable levels of gene expression (Fig. 3a and d). Likewise, MCT8 exhibited a pattern of decreasing expression (Fig. 3b). However, this decrease was prolonged and gradual. MCT10 displayed a very complex expression pattern (Fig. 3c), highlighted by a 2-fold expression increase from NF stage 59/60. This level of expression then remained stable for the remaining developmental stages examined. Transporter LAT1 was expressed near the limit of detection from NF 56 to 59 but was significantly up-regulated 4-fold by NF stage 63 (Fig. 3d). Several transporters, including OATP4A1, OATP1C1, and OATP4C1, were expressed at or below the limit of detection throughout metamorphosis (data not shown).

3.4. Brain

Only two transporters, MCT8 and OATP1C1, showed expression patterns that significantly varied over developmental time. Transporter MCT8 exhibited a significant increase in expression at NF stage 54, followed by relatively stable expression (Fig. 4a). In contrast, OATP1C1 (Fig. 4b) displayed a complex expression pattern with peak expression at NF stage 56. No significant expression pattern was observed for MCT7 (78.4 ± 22.1–69.8 ± 16.6 copies/ng total RNA), MCT10 (542.4 ± 261.8–236.5 ± 116.8 copies/ng total RNA), LAT1 (3417.6 ± 1824.4–2271.9 ± 377.9 copies/ng total RNA), OATP4A1 (63.5 ± 41.6–90.1 ± 25.3 copies/ng total RNA) and OATP1B3 (100.7 ± 57.8–28.9 ± 13.5 copies/ng total RNA). Data for these transporters is not shown. Additionally, expression levels for transporter OATP4C1 were at or below the detection limit (data not shown).

3.5. Gill

In the gill, expression of OATP1C1, OATP4A1, OATP1B3 and MCT8 transporters remained at a low level during NF stages 56–60 but significantly increased during NF stage 61 (Fig. 5e, f, h and b). Transporter MCT10 also exhibited a significant increase in expression at NF stage 61, however this up-regulation occurred gradually throughout NF 58–61 (Fig. 5c). Conversely, OATP4C1 was increasingly expressed with a peak at NF stage 60 followed by a significant decrease of expression at NF stage 61 (Fig. 5g). A more dynamic expression pattern was observed with MCT7 and LAT1 genes. Both transporters displayed an increase in expression during NF stages 56–58 (Fig. 5a and d), with a dramatic decrease in expression during NF stage 59. This decrease was followed by a modest increase in expression through NF stage 61.

3.6. Tail

Distinct and unique gene expression patterns were observed in the resorbing tail. The most dramatic of these was exhibited by the transporter MCT8, which increased expression 11-fold from NF stages 60 to 64 (Fig. 6b). MCT10 expression also showed a significant increase between NF stages 60 and 61, with stable expression for the remainder of tail resorption (Fig. 6c). Interestingly, MCT10 tail expression levels were 8-fold higher than any other tissue/gene combination examined. An increase in expression of OATP1C1 (Fig. 6e) was also observed from below the detection limit at NF stage 60 to fluctuating levels in later development. Transporter OATP4A1 (Fig. 6f) also exhibited low, fluctuating, expression levels.

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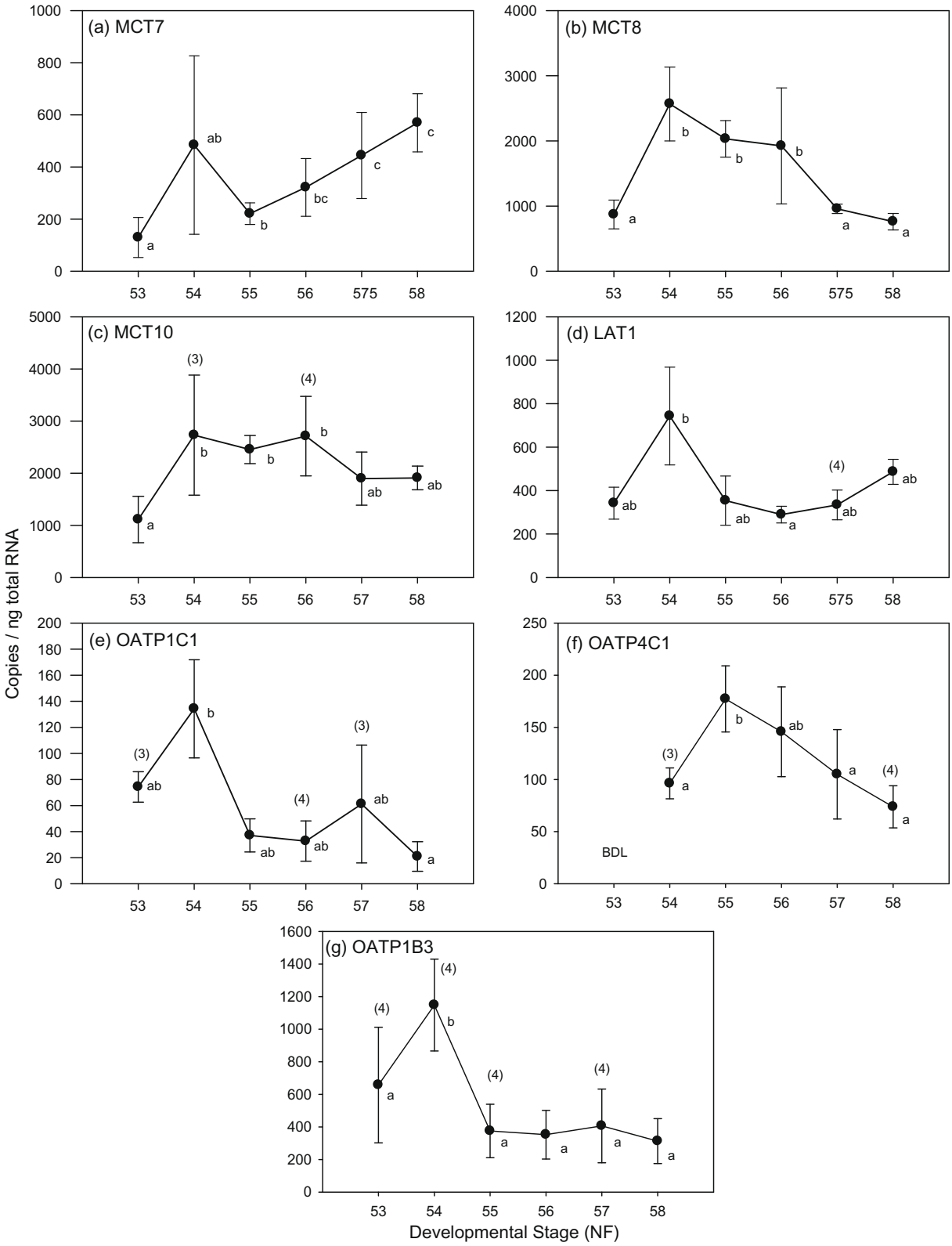

Fig. 1. Survey of TH transporter expression in the metamorphosing *Xenopus tropicalis* hind limb. Gene expression was quantified using qRT-PCR and normalized to ng of total RNA. Values are reported as mean ± SD with a sample size of $n = 5$, unless otherwise noted (n). Letters differentiate expression levels that are significantly different ($p < 0.05$). Results of OATP4A1 were not shown do to its stable expression (see text). BDL; below detection limit.

Stable expression of the LAT1 transporter (Fig. 6d) was observed throughout development, with a significant increase in expressing during NF stage 62. MCT7 (Fig. 6a) exhibited a dynamic expression pattern with a significant up-regulation of expression throughout tail metamorphosis with peak levels at NF stage 62/63. Gene expression was then significantly decreased during NF stage 64. OATP4C1 did not show a statistically significant expression pattern. However, expression levels varied from 52.2 ± 12.5 to

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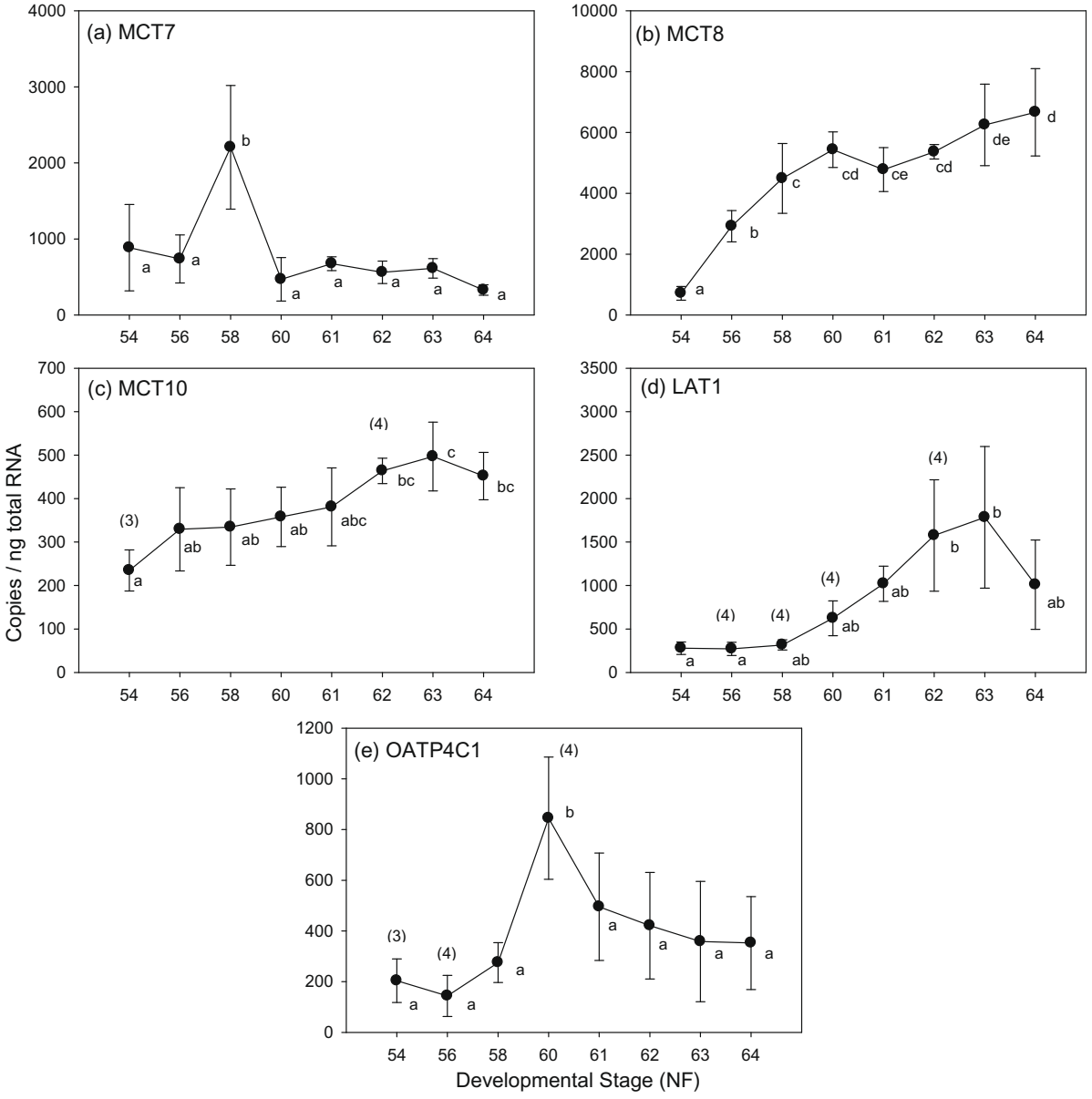

Fig. 2. Survey of TH transporter expression in the metamorphosing *Xenopus tropicalis* kidney. Gene expression was quantified using qRT-PCR and normalized to ng of total RNA. Values are reported as mean ± SD with a sample size of $n = 5$, unless otherwise noted (n). Letters differentiate expression levels that are significantly different ($p < 0.05$). Results of OATP4A1 were not shown do to its stable expression (see text). Transporters OATP1C1 and OATP1B3 were expressed at or below limit of detection.

109.3 ± 39.8 copies/ng total RNA (data not shown). Additionally, expression levels for transporter OATP1B3 were at or below the detection limit (data not shown).

4. Discussion

Transport of TH across the plasma membrane has been shown to be a rate-limiting step in TH metabolism and, presumably, in the expression of thyroid hormone activity (Hennemann et al., 1986). Because TH transporters have shown similar transporting capabilities across species (Pizzagalli et al., 2002; Sugiyama et al., 2003; Tohyama et al., 2004), we assumed these transporters were likely to be functioning in the same manner in *Xenopus*. This study examined the developmental expression patterns of potential TH transporters in an effort to understand what role, if any, differential TH transporter expression may play in regulating or facilitating TH-induced metamorphosis. An additional gene with unknown substrate transport capabilities (MCT7) was also included in this study. In a preliminary gene array experiment, MCT7 was shown to be expressed in a TH-sensitive manner in the pituitary. In each tissue examined, the expression of MCT7 demonstrated significant differences among developmental stages. These expression patterns were similar to the patterns of known TH transporters examined in this study. MCT7's dynamic developmental expression makes it especially appealing for future studies to determine its transport substrates and potential role in metamorphosis. While this discussion will focus on the expression patterns of TH transporters in the context of their potential role in mediating TH bioavailability to specific tissues, it is important to emphasize that many TH transporters are capable of transporting a wide range of substrates in addition to THs.

The hind limb is one of the first tissues to undergo TH-induced metamorphic changes. Continued limb growth and differentiation

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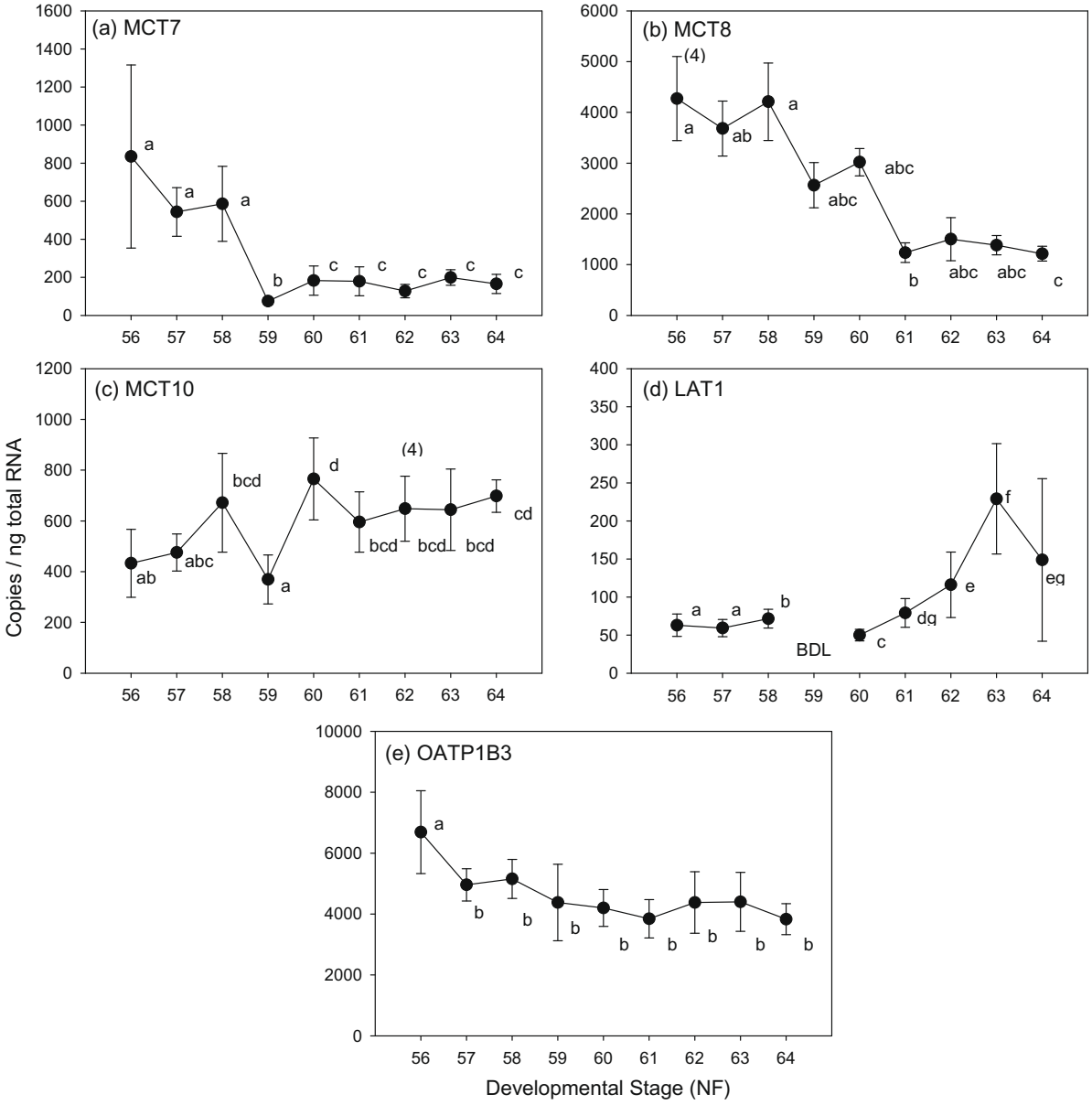

Fig. 3. Survey of TH transporter expression in the metamorphosing *Xenopus tropicalis* liver. Gene expression was quantified using qRT-PCR and normalized to ng of total RNA. Values are reported as mean ± SD with a sample size of $n = 5$, unless otherwise noted (n). Letters differentiate expression levels that are significantly different ($p < 0.05$). Transporters OATP4C1, OATP1C1 and OATP1B3 were expressed at or below limit of detection. BDL; below detection limit.

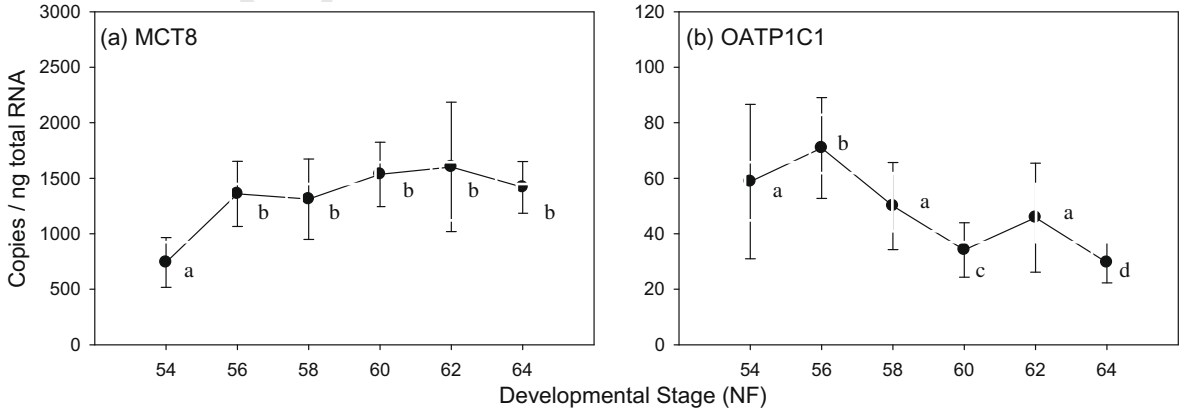

Fig. 4. Survey of TH transporter expression in the metamorphosing *Xenopus tropicalis* brain. Gene expression was quantified using qRT-PCR and normalized to ng of total RNA. Values are reported as mean ± SD with a sample size of $n = 5$. Letters differentiate expression levels that are significantly different ($p < 0.05$). Results of MCT7, MCT10, LAT1, OATP4A1 and OATP1B3 were not shown do to their stable expression (see text). Transporter OATP4C1 was expressed at or below limit of detection.

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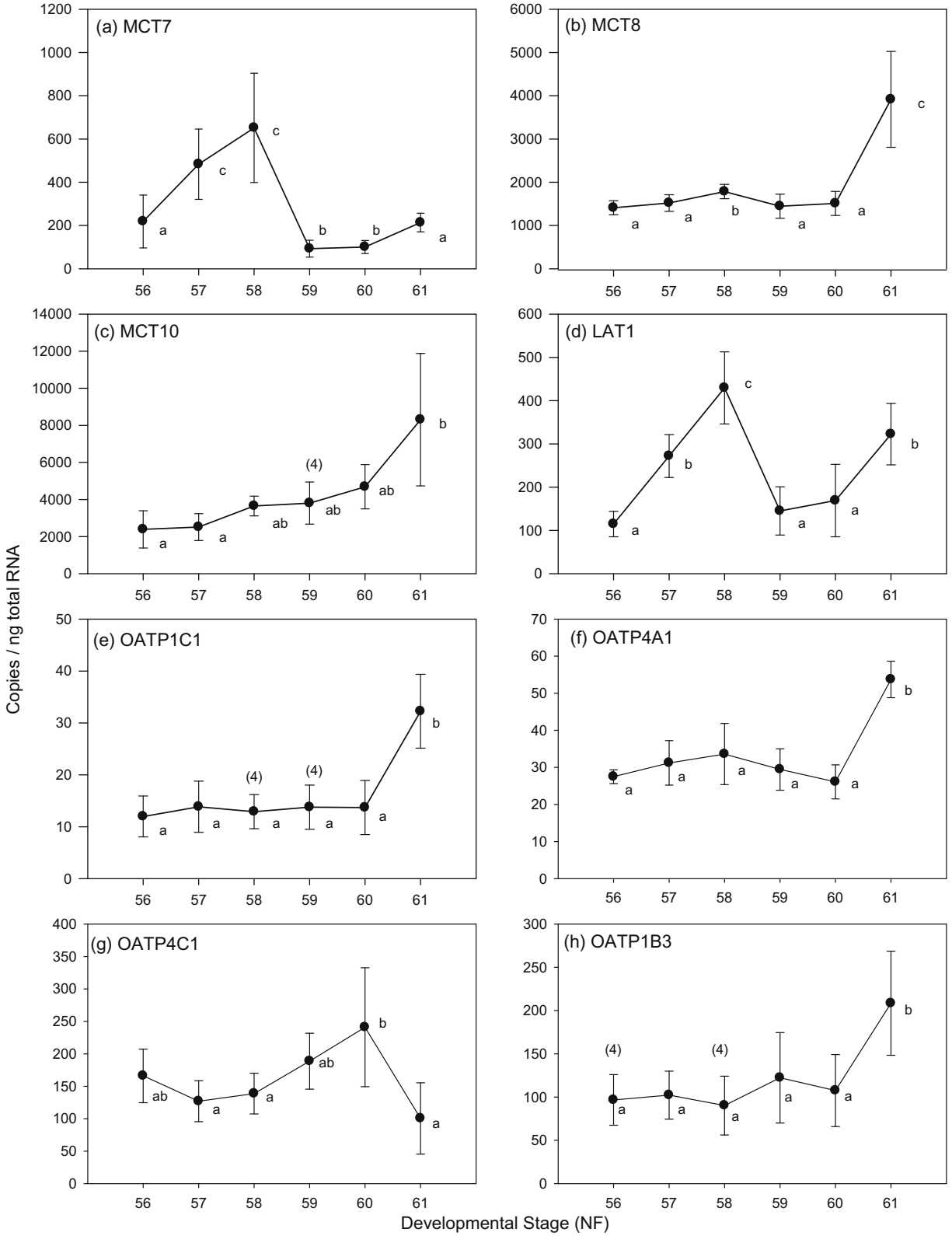

Fig. 5. Survey of TH transporter expression in the metamorphosing *Xenopus tropicalis* gill. Gene expression of MCT7, MCT8, MCT10, LAT1, OATP1C1 and OATP4A1 were quantified using qRT-PCR and normalized to ng of total RNA. Values are reported as mean ± SD with a sample size of $n = 5$, unless otherwise noted (n). Letters differentiate expression levels that are significantly different ($p < 0.05$).

requires continuous exposure to endogenous TH from NF stage 53 (Brown et al., 2005) until NF stage 58 (Elinson et al., 1999). Remarkably, these changes begin before a significant rise in plasma TH concentrations is observed (Leloup and Buscaglia, 1977). The limb's ability to grow and develop in low TH conditions has been widely attributed to the presence of high concentrations of the TH activating D2 enzyme (Becker et al., 1997; Cai and Brown, 2004; Huang et al., 2001). Strong expression levels of thyroid

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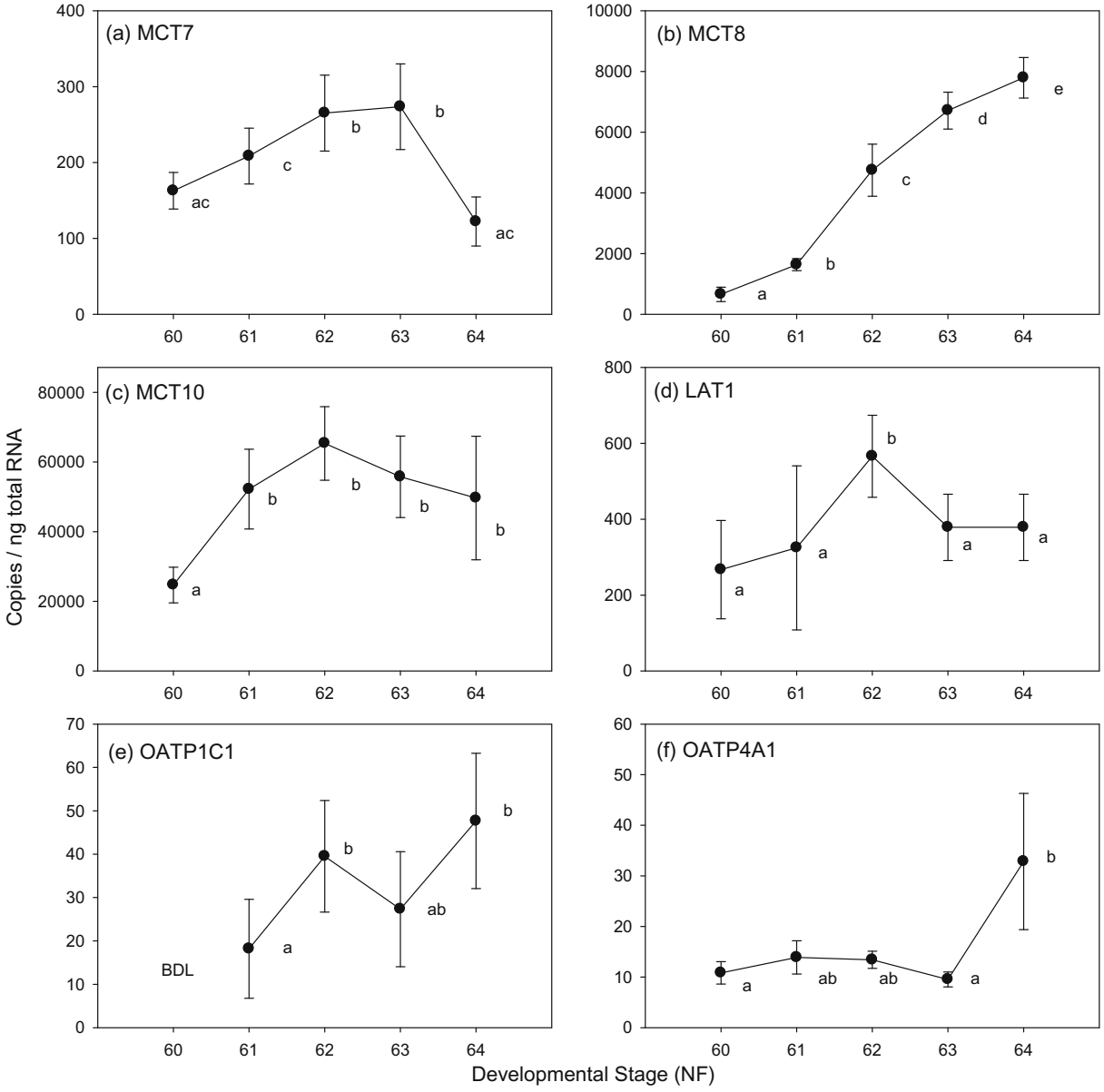

Fig. 6. Survey of TH transporter expression in the metamorphosing *Xenopus tropicalis* tail. Gene expression was quantified using qRT-PCR and normalized to ng of total RNA. Values are reported as mean ± SD with a sample size of $n = 5$. Letters differentiate expression levels that are significantly different ($p < 0.05$). Results of OATP4C1 were not shown do to its stable expression (see text). Transporter OATP1B3 was expressed at or below limit of detection. BDL; below detection limit.

receptors TRα and RXRα in the limb are also presumed to enable early TH-induced gene expression (Cai and Brown, 2004). Together, these conditions prepare the limb for a rapid response when exposed to TH. However, sufficient amounts of TH must still be transported into the tissue before any TH-induced changes can occur.

Given low plasma concentrations of TH in the blood during the early stages of prometamorphosis, TH transporting proteins may play an important role in mediating limb development. If these proteins helped facilitate TH bioavailability, we hypothesized that an increase in expression would be observed during NF stages 54–58. Two transporters, MCT8 and MCT10, show particularly interesting expression patterns during limb TH-sensitive stages. Both transporters show robust expression levels throughout limb development with a significant up-regulation during NF stages 54–56. Peaks in the expression of OATP4C1, OATP1B3, OATP1C1 and LAT1 transporters were also observed during NF 54/55. Additionally, MCT7 displayed a gradual increase in expression throughout development.

Copy numbers of the transporter genes examined in the hind limb varied; MCT8 and MCT10 transporter expression was observed at levels 2–10 times greater than other genes. MCT8, and to a lesser extent MCT10, are known to be highly specific TH transporters capable of rapid and efficient transport (Friesema et al., 2003, 2008). Collectively, these observations are consistent with transporters MCT8 and MCT10 playing a role in TH transport into the hind limb and are thus influencing metamorphic processing.

Tissues that undergo death or resorption in response to TH typically undergo their metamorphic changes during the climax of metamorphosis. During these stages, plasma TH concentrations are at their peak (Leloup and Buscaglia, 1977). Tail resorption occurs during this phase, with noticeable morphological changes occurring at NF stage 62 (Nieuwkoop and Faber, 1994). Several different mechanisms have been attributed to controlling the timing of the TH-induced response and preventing it from occurring prematurely due to the presence of endogenous TH. One example is the expression of the TH inactivating enzyme D3. Several studies

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have shown that the larval tail expresses low levels of D3 throughout early stages of metamorphosis (Kawahara et al., 1999; St. Germain et al., 1994). Presumably, D3 would serve as protection from the tail undergoing metamorphosis under low concentrations of TH. At the onset of tail resorption, D3 expression declines and D2 is up-regulated (Cai and Brown, 2004; Huang et al., 2001). Temporal expression of thyroid hormone receptors in the tail has also been associated with the timing of tail resorption (Wang and Brown, 1993).

We hypothesize another mechanism that could prevent precocious tail metamorphosis might involve denying transport of TH across the plasma membrane. For example, expression of TH transporters could be delayed until the desired developmental time point was reached. The developmental expression pattern of MCT8 which exhibited a 4-fold increase in expression over five developmental stages, lends support to this hypothesis. A significant and dramatic up-regulation of MCT10 was also observed from NF stages 60 to 61 and remained stable for the remaining life of the tail. Other transporters (MCT7, LAT1, OATP1C1 and OATP4A1) were expressed at comparatively low copy numbers but showed increased expression coinciding with tail resorption.

The resorbing gill is another tissue ideal for assessing potential roles of TH transporters in mediating metamorphic events. Gill resorption occurs slightly earlier than tail resorption, and is complete by NF stage 62. This death/resorption plan coincides with the metamorphic climax of TH concentrations. Interestingly, the gill is widely reported to lack the activating D2 enzyme. Because the gill is unable to produce its own active T_3 hormone, it is has been suggested that the gill metamorphoses once sufficient T_3 concentrations have been reach in the blood (Huang et al., 2001).

Our results suggest that differential expression of TH transporters may also play a role in increasing gill tissue concentration of THs. The most prominent transporter observed in the gill was MCT10. MCT10 exhibited a slow, but significant increase in expression during NF stages 58–61; corresponding to stages associated with gill death and resorption. An increase in expression culminating at NF stage 61 was also observed for MCT8, OATP1C1, OATP4C1 and OATP1B3. However, these transporters were not expressed in as high quantities as MCT10. Interestingly, LAT1 and MCT7 showed a significant increase in expression from NF stages 56 to 58.

We examined expression of TH transporters in the brain throughout the metamorphic process. Surprisingly, only two transporters (MCT8 and OATP1C1) showed expression patterns that significantly varied over developmental time. MCT8 exhibited a 2-fold increase in expression from NF stage 54 to NF stage 56, followed by stable expression. In contrast, OATP1C1 expression levels fluctuated throughout developmental time. However, the comparatively low expression levels of OATP1C1 (tens of copies per ng total RNA) makes this transporter unlikely to be biological significance. These results suggest indicate that TH transporter do not show dynamic expression pattern in whole brain homogenate. These results should be interpreted with some degree of caution. The brain is a very complex tissue and based on the mammalian literature there could be important region specific expression that would not been observed by using whole brain homogenates. In future studies it may be more appropriate to use a method such as *in situ* hybridization, a technique capable of providing insight into brain region specific expression patterns. It is also important to note that the hypothalamus was removed from the brain in these experiments. The intent was to examine TH transporter expression in the hypothalamus, however our inability to extract sufficient levels of high quality RNA prevented this analysis.

In both the remodeling liver and the kidney, transporter gene copy numbers were profoundly varied. Many transporter genes were observed in concentrations of only tens or hundreds of copies per ng of total RNA. The functional significance of these transporters in facilitating metamorphic change is questionable. However, both the liver and kidney exhibited expression of MCT8 and MCT10 at copy numbers that were orders of magnitude larger than other transporters. The strong correlation between high levels of MCT8/MCT10 expression and metamorphic change in these resorbing tissues are consistent with these transporters playing a role in influencing the metamorphic timing of the gill and tail.

Metamorphic changes that convert the larval pronephros kidney into a mesonephros kidney gradually occur during premetamorphosis and finish by metamorphic climax (Nieuwkoop and Faber, 1994). However, specific developmental windows of TH sensitivity have not been clearly defined. Many of the changes associated with kidney remodeling have been attributed to the larval switch from ammonotelism (ammonia excretion) to ureotelism (urea excretion). Presumably, this would coincide with a change in the expression profile and distribution of organic anion transporters to facilitate the elimination of various compounds into the urine.

Analyzing the potential role of TH hormone transporters in mediating kidney TH bioavailability is complicated by the promiscuous nature of transporters. From a TH-focused view, the gradual up-regulation of MCT8 and MCT10 expression observed in this study may correspond with the gradual pace of kidney metamorphosis. Because amphibian kidneys lack D2 activating enzymes (Becker et al., 1997), the high T_3 affinity of MCT8 and MCT10 make these transporters especially interesting biologically. Other transporters with more promiscuous substrate capabilities also showed statistically significant differences in developmental gene expression. One such TH transporter, OATP4C1, has also been shown to facilitate the elimination of drugs into the urine (Mikkaichi et al., 2004). OATP4C1 expression remained at a relatively low but stable level of expression with the exception of a significant increase during NF stage 60. Interestingly, MCT7 also showed a constant level of expression with the exception of a single stage, NF stage 58. While these transporters may play a role in increasing TH bioavailability, it is likely they also play a role in maintaining generally physiological function. Further characterization of these proteins would be required to establish true biologic function.

The metamorphic remodeling changes that occur in the larval amphibian liver begin during metamorphic climax. The timing of liver remodeling has been hypothesized to be controlled by expression of the inactivating D3 enzyme. Several experiments have shown that the liver has high D3 activity during pre- and prometamorphosis, possibly preventing premature T_3 accumulation (Becker et al., 1997; Kawahara et al., 1999). Like the kidney, the liver also lacks activating D2 enzymes (Becker et al., 1997). We anticipated seeing an increase in the concentration of TH transporters corresponding to developmental stages associated with liver metamorphic change.

Surprisingly, the high level of MCT8 expression during NF stages 56–58 was followed by a significant decrease of expression for the remainder of metamorphosis. It is possible that MCT8 works in concert with other transporters, and is only responsible for the initial supply of TH to the tissue. Late MCT8 expression may then be decreased to stable, homeostatic levels required for normal physiological function. MCT7 expression followed a similar pattern, with a significant decrease at NF stage 59 followed by relatively stable expression. Cellular expression of MCT10 remained low during NF stages 56–58, and gradually but significantly increased in concentrations throughout metamorphosis. This compliments the expression of MCT8 and MCT7, possibly working together to maintain a specific cellular concentration of TH.

It is important to note that the TH transporter with the highest expression levels in the liver is OATP1B3. Even though OATP1B3 has been reported as capable of transporting TH, the primary function of this transporter is believed to be in facilitating metabolism

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in the liver through transport (Abe et al., 2001; Smith et al., 2005). It is therefore no surprise that this protein is expressed in such high concentrations. OATP1B3 was expressed at statistically higher concentrations during NF stage 56, and then down-regulated to a constant basal level of expression. It is unclear what biological significance this period of high expression may have. Further, it is difficult to guess if OATP1B3 plays a significant role in facilitating metamorphosis.

Both the liver and the kidney play a vital physiological role in general metabolism and excretion of waste. Another possible role for TH transporters in these tissues may be to assist in maintaining physiological levels of TH in the blood via control of TH excretion. This process may be especially important in decreasing the circulating concentrations of TH in the blood after metamorphic climax has been reached. If transporters were functioning under this mechanism, we expected to see elevated levels of expression during the final stages of metamorphosis, especially after the tissue had completed its metamorphic change. In the kidney, the continued elevated expression of MCT8 and MCT10 transporters during NF stages 62–64 followed our anticipated expression pattern. Interesting, LAT1 expression peaked at NF stage 62/63. In the liver, both MCT10 and LAT1 displayed elevated expression patterns at the end of metamorphosis. Together, these expression patterns are consistent with the hypothesis that these transporters may be playing a role in TH transport into the kidney and liver and, therefore, influencing TH metabolism and excretion after metamorphic climax.

The results of this study were interpreted under the assumption that TH transport is occurring in a unidirectional fashion resulting in a net influx into the cell. Our results are complicated by issues of bidirectional flow and efflux. Indeed, both MCT8 and MCT10 have been shown to have bidirectional transport abilities (Friesema et al., 2008). It is also important to emphasize that these results were obtained from mRNA transcripts. mRNA expression levels do not always directly relate to protein levels, nor do they demonstrate physiological function. Studies of this nature seldom provide direct evidence; however, they do provide the basis for generating testable hypotheses. Collectively, this survey of TH transporter expression levels throughout metamorphosis provides a basis to suggest that thyroid hormone transporters may play an important role in metamorphic processes. More definitive evidence, perhaps through targeted gene knock-out experiments, would be required to determine the role of these proteins in metamorphosis.

Disclaimer

This paper has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Mention of trade names of commercial products does not constitute endorsement/recommendation of use.

5. Uncited reference

Q1 Yanase et al. (2008).

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