Beyond Synergy: Corticosterone and Thyroid Hormone Have Numerous Interaction Effects on Gene Regulation in *Xenopus tropicalis* Tadpoles

Saurabh S. Kulkarni and Daniel R. Buchholz

Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221

Hormones play critical roles in vertebrate development, and frog metamorphosis has been an excellent model system to study the developmental roles of thyroid hormone (TH) and glucocorticoids. Whereas TH regulates the initiation and rate of metamorphosis, the actions of corticosterone (CORT; the main glucocorticoid in frogs) are more complex. In the absence of TH during premetamorphosis, CORT inhibits development, but in the presence of TH during metamorphosis, CORT synergizes with TH to accelerate development. Synergy at the level of gene expression is known for three genes in frogs, but the nature and extent of TH and CORT cross talk is otherwise unknown. Therefore, to examine TH and CORT interactions, we performed microarray analysis on tails from *Xenopus tropicalis* tadpoles treated with CORT, TH, CORT+TH, or vehicle for 18 h. The expression of 5432 genes was significantly altered in response to either or both hormones. Using Venn diagrams and cluster analysis, we identified 16 main patterns of gene regulation due to up- or down-regulation by TH and/or CORT. Many genes were affected by only one of the hormones, and a large proportion of regulated genes (22%) required both hormones. We also identified patterns of additive or synergistic, inhibitory, subtractive, and annihilatory regulation. A total of 928 genes (17%) were regulated by novel interactions between the two hormones. These data expand our understanding of the hormonal cross talk underlying the gene regulation cascade directing tail resorption and suggest the possibility that CORT affects not only the timing but also the nature of TH-dependent tissue transformation. (*Endocrinology* 153: 5309–5324, 2012)

Glucocorticoids (GC) play critical roles throughout vertebrate life history (1, 2). Developmental roles include maturation of various organs like lungs (3–7), brain (1, 6–9), intestine (6, 7, 10, 11), stomach (12), pancreas (13), and kidney (7, 11). In adults, GC affect cardiovascular physiology, metabolism, immune function, neural function, behavior, and reproduction (1, 2, 14). Peak levels of GC are reached at birth, hatching, or metamorphosis in mammals, birds, fish, amphibians, and reptiles (2, 6, 7, 11, 15–17). Thyroid hormone (TH) also plays a critical role in development, differentiation, and growth in all vertebrates (18, 19). In particular, mammalian studies have shown that the lack of TH in early human development results in growth disturbances and severe mental retardation, a disease called cretinism (18).

Because TH and GC are developmental hormones acting on some of the same organs, one may expect some degree of TH and GC interaction. Indeed, cross talk between TH and corticosterone (CORT; the main GC in mice and frogs) has been shown *in vivo* and *in vitro*. For instance, intestinal deficiencies caused by the down-regulation of duodenal phosphatase enzyme in hypophysectomized rat can be repaired by the addition of both TH and CORT, which act synergistically to up-regulate the enzyme to its normal levels (20). TH and CORT also interact to regulate hypothalamic genes like brain-derived neurotrophic factor and neuropeptide Y in chicken that play important roles in maintaining energy homeostasis (21). Furthermore, previous studies found a synergistic up-regulation of the Kruppel-like tran-

Abbreviations: CORT, Corticosterone; GC, glucocorticoid; GO, gene ontology; GRE, glucocorticoid response element; HRE, hormone response element; iGRE, inhibitory GRE; KLF9, Kruppel-like transcription factor; NF53, Nieuwkoop and Faber stage 53; TH, thyroid hormone; TRE, TH response element.

scription factor (KLF9) by TH and CORT in a mouse hippocampal cell line (HT-22) (22). They identified regions of the *KLF9* gene in mouse that support synergistic gene activation in transfection assays and that exhibit hyperacetylation of histones upon hormone treatment (22, 23). By far the best-studied system for TH and CORT interactions is the endocrine control of frog metamorphosis (24). Even though amniotes do not undergo metamorphosis, the timing of tadpole metamorphosis and birth in mammals share conserved mechanisms of endocrine control (25–28). In particular, the transition from an aqueous to a terrestrial environment in fetuses and tadpoles is associated with adaptive organ remodeling regulated by hypothalamus-pituitary-thyroid and hypothalamus-pituitary-adrenal axes (25–28).

TH and CORT interactions are essential for frog metamorphosis. TH plays a key role in frog metamorphosis (24, 29–33), in that TH is necessary and sufficient to initiate metamorphosis. However, TH alone is not sufficient to complete metamorphosis in the absence of CORT (29, 32–35), and the mechanistic basis of this CORT requirement is not known. Even though CORT does not have metamorphic actions independent of TH (23, 24, 33, 36), CORT accelerates metamorphosis during the prometamorphic period when TH is present and inhibits development during the premetamorphic period when TH is absent (23, 37–46). Little is known about how premetamorphic CORT inhibits development, but CORT and TH accelerate the rate of tail resorption to a greater extent together compared with their independent effects *in vitro* as well as *in vivo* (38, 40).

Several mechanisms underlying TH and CORT synergy during metamorphosis have been identified. First, CORT can amplify the effect of TH on induction of genes like *TRβ* (TH receptor-β), *TRα* (TH receptor-α), deiodinase 2, and deiodinase 3 (23, 38, 47, 48). At least for TRβ, CORT can bind and positively regulate its expression via a glucocorticoid response element (GRE) in the promoter region (23, 36, 38). CORT can increase deiodinase 2 and decrease deiodinase 3 enzyme activity, which activates and degrades TH, respectively, increasing intracellular concentrations of T_3, the active form of TH (37, 46). Second, recent studies by Bonett *et al.* (38, 47) showed that the transcription factor *KLF9*, which is a TH direct-response gene, is also a CORT direct-response gene. Importantly, CORT and T_3 can synergize to cause a superinduction of KLF9 expression both in *X. laevis in vivo* and in frog cell cultures *in vitro*. In addition, their experiments revealed that KLF9 might regulate TRβ expression. Therefore, stress-induced production of CORT can increase KLF9 expression, which in turn can increase TRβ expression,

ultimately resulting in increased rate of tissue transformation (49, 50).

Despite the above-mentioned studies, knowledge of the potential range of TH and CORT interactions on gene regulation is still largely unknown. Also, much work has been carried out on the gene regulation cascade induced by TH to accomplish metamorphosis (24, 33, 51), but the actions and molecular underpinnings of CORT's role during metamorphosis have yet to be elucidated. Subtractive hybridization and microarray technology have proved instrumental in discovering genes and molecular pathways underlying TH action not only during metamorphosis of whole tadpoles but also of individual tissues, such as brain, tail, hind limbs, skin, and intestine (24, 52–64). Recent studies have tested some of these genes in isolation to tease out their effects during metamorphosis (24, 47, 65, 66). Identification of the genes regulated by CORT independently and in synergy with TH is required to better understand metamorphosis in anurans and postembryonic development in general. Therefore, we performed microarray experiments on tails of *X. tropicalis* tadpoles treated with TH and/or CORT.

Materials and Methods

Animals, experimental design, and RNA extraction

Tadpoles of *X. tropicalis* were purchased from Xenopus Express (Brooksville, FL). Tadpoles were reared at 26 C until they reached Nieuwkoop and Faber stage 53 (NF53) (67). At NF53, tadpoles were subjected individually in 4-liter tanks to one of four treatments for 18 h (n = 6): 1) control (ethanol), 2) 100 nM CORT (HPLC grade; Sigma, St. Louis, MO), 3) 50 nM T_3 (HPLC grade; Sigma), and 4) 100 nM CORT + 50 nM T_3 at 26 C. Tails were dissected and snap frozen from each tadpole. RNA was extracted from frozen tail samples using Trizol (Invitrogen, Carlsbad, CA), and high quality of total RNA was shown by capillary electrophoresis using a BioAnalyzer (Agilent Technologies, Santa Clara, CA). RNA samples from all four treatments were tested with RT-PCR to verify the expected changes in expression of TRβ and KLF9 (data not shown).

Treatment and condition justifications

We chose the 100-nM dose for CORT because it elevated whole-body CORT within the physiological range in tadpoles and caused phenotypic and gene expression changes in tadpoles and frogs (33). We chose 50 nM treatment for TH because earlier studies showed that 50 nM but not 5 and 10 nM treatments caused significant phenotypic and gene expression changes in the tails of *X. laevis* tadpoles (33). We chose the duration of treatment to identify mainly direct response genes. Some direct response genes are detectable by 6 h (62), and it is possible that some of these genes will be transiently expressed for less than 1 d. However, most known direct response genes are strongly induced by 24 h (24). Indirect response genes are also likely to be represented to some extent before 24 h but often are not detected until 2 or more

days. To achieve a compromise time point to enrich for direct response genes, a treatment for 18 h was chosen.

Because of potential interference from endogenous hormones, it is possible that the hormone regulation of some genes may be misidentified, such that some genes induced by the TH treatment may actually require CORT and vice versa. We avoided the use of chemicals to block TH because of their known independent effects on gene regulation by themselves (68, 69). The independent effects on gene regulation of chemicals that block CORT production or action have not been examined. To reduce the effects of endogenous hormones, the developmental stage NF53 was chosen because the endogenous TH and CORT levels are minimal at this stage (24, 70). In addition, to minimize stress-induced CORT production caused by rearing conditions, the tadpoles were treated individually in 4-liter tanks. Furthermore, the tail does not undergo resorption until the concentrations of both hormones reach their peak levels at the climax of metamorphosis (NF62) (24); therefore, the low concentration of these hormones at NF53 is unlikely to affect gene expression in the tail.

We chose the tail because it is easy to harvest but more importantly because *in vitro* tail tip cultures revealed that the synergistic effect of TH and CORT on tail shrinkage occurs at the level of the tissue (38). We expect our results will apply to other tissues because thyroid hormone receptor and glucocorticoid receptor are expressed in all tissues examined. Also, one previous gene (*KLF9*), shown to be a direct response gene of TH and CORT, is expressed not only in the tail but also all tissues examined (38, 54, 55, 57, 62). Furthermore, like the tail, many tissues experience apoptosis during metamorphosis and may have similar underlying endocrine regulation of this process (24). Thus, the regulation of genes identified here may be similarly regulated in other tissues.

Microarray analysis

Three nonpooled samples from each treatment were used, and the microarray analysis was carried out at the Cincinnati Children's microarray facility using the Affymetrix *Xenopus tropicalis* GeneChip (Affymetrix 1.0; Santa Clara, CA) representing 59,021 probe sets. Bioinformatics analysis was performed using GeneSpringGX software (version 11.02; Agilent Technologies). We created a gene level experiment, in which results from all probe sets representing the same gene were averaged and used as a single entity. As a quality control, all genes were checked by principal components analysis for grouping by treatments. Genes were then filtered for signal intensity values (genes for which at least one sample had between 25 and 98% of the observed signal intensity were selected), which allowed us to remove very low signal values or those that have reached saturation. Furthermore, the gene list was filtered for error (genes with coefficient for variation up to 90% were selected). The genes passing these quality controls were then analyzed by ANOVA at $\alpha = 0.05$ followed by *post hoc* Tukey-Kramer to find pairwise significant differences between treatments. k-means clustering on genes in sections of Venn diagrams was used to identify groups of genes with similar expression patterns. Gene ontology (GO) analysis was carried out using GeneSpring.

RT-PCR analysis

We used RT-PCR analysis to find examples of gene expression patterns representing some of the clusters identified by our analysis of the microarray data. We selected genes that were differentially regulated and highly expressed compared with control or showed little variation in expression in the data analysis. To perform RT-PCR, we used RNA from the same samples submitted for the microarray experiment. RNA was subjected to cDNA synthesis (high capacity cDNA reverse transcription kit; Applied Biosystems Inc., Foster City, CA) as per the manufacturer's protocols. PCR (Takara Bio Inc., Mountain View, CA) on each cDNA sample (n = 3) was carried out using primers for each gene (Table 1). The housekeeping gene *rpL8* was used to control for variation among samples from the RNA extraction procedure (17, 53, 56).

TABLE 1.   Primer pairs used for RT-PCR

Gene (Unigene)	Cluster	Strand	Primer sequence (5'-3')
Transcribed locus (Str. 8302)	A1	Forward	TTGCTGAATGCCCCCAATG
		Reverse	CCCACCCACCACACAAACATAC
KLF9 (Str. 61607, 58576)	B1	Forward	TATGGACTACAGCCCCGAAC
		Reverse	TGGACCTTTTGGACCTTTTG
Thyroid hormone receptor-β (Str. 52012)	C1	Forward	AAAATGGAGGACTCGGTGTG
		Reverse	AAAAAGTGACGGAGGCAATG
Glutamate carboxypeptidase 2-like (Str. 61805)	D1	Forward	GCTCGCGGAACCTGCTGACTTT
		Reverse	GCTCCCCCTCTGGGGTTCCAG
Solute carrier family 1 (Str. 1713)	F1	Forward	ACAGACTCCCGTGCCCACGT
		Reverse	CGAGCTTGCCGAGAGCGGAG
MICAL like 2 (Str. 31608)	F1	Forward	GCCCTGCAGCAGTGGTGYAARCA
		Reverse	ACCCGCAGCTTCACCATRTCYTC
Hypothetical LOC496814 (Str. 16451)	G1	Forward	CGGGAAAAAACTTACTGGAGAGC
		Reverse	CTGGATAAATGGAGTGCTGGCTAC
ZBTB16 (Str. 44724)	G1	Forward	CAGTGGGTATGGCACAAGAATGAG
		Reverse	TTTGGGTTTGGGGGGAAAG
Transcribed locus (Str. 34945)	G1	Forward	AGGGCAGTGGAAACAGTAACTCAC
		Reverse	ATCGGGTCAAGTCGGTATTATCC
RpL8		Forward	CGTGGTGCTCCTCTTGCCAAG
		Reverse	GACGACCAGTACGACGAGCAG

TABLE 2. Comparison of expression level changes in Das *et al.* (59) (48 h) and in the TH treatment of the current study (18 h) for the genes in Table 1 of Das *et al.* (59) that were represented on the Affymetrix *X. tropicalis* GeneChip

| | | Fold up-regulation | |
Unigene	Gene	Das *et al.* (59)	Current study
Str.32442	Fibroblast activation protein-α	71.7, 51.6	5.2
Str.53813	Gene B MAM domain	62.1, 40.2	3.44
Str.41312	MMP-13 (collagenase 3)[a]	53.6, 16.4, 9.6	2.50
	CRF binding protein	51.6	1.10
Str.6148	Gap junction channel protein-β 6	34.3	NC
Str.27324	RAS-like GTP-binding protein	16.7	5.39
Str.24553	Arginase type I	12.8	1.39
Str.65147	Hyaluronoglucosaminidase 2	11.8	2.47
Str.25784	Sox 4[a]	11.7	4.21
	RUNX1	10.9	1.49
Str.66828	Glycine dehydrogenase	10.2	3.16
Str.34914	Solute carrier family 43	10.1	1.51
Str.65170	Glutamine synthase	9.9	1.67
Str.26977	α-Aspartyl dipeptidase (gene D)	7.4, 5.7	1.82
Str.20796	MMP-2 (collagenase 4)	7.3	2.1
Str.21804	Galectin 1	7.2	2.34
Str.37212	Arginase type II	6.9	4.96
Str.55321	Biglycan	6.9	1.28
Str.84561	FGF 9 (glia-activating factor)	6.7	1.89
Str.53061	Iodothyronine deiodinase type III	6.7, 6.2	6.37
Str.41102	C/EBP δ-1	6.3	2.62
Str.27804	Dipeptidylpeptidase 4	6	1.57

[a] Affymetrix *X. tropicalis* GeneChip does not list Unigene numbers for these genes. NC, No change in expression.

Comparison of microarray data with previous microarray experiments

We compared the genes regulated by TH found by microarray analysis in Das *et al.* (59) (Table 2) and Buchholz *et al.* (57) to our microarray data. We also compared our TH response gene list to that of overlapping TH response genes in *X. laevis* tails among three array studies [Helbing *et al.* (62), Das *et al.* (59), and Searcy *et al.* (64)] as identified by Searcy *et al.* (64).

Results

To understand the molecular pathways initiated during frog metamorphosis by interaction of TH and CORT, we performed global gene expression analysis on tails from tadpoles treated with CORT and/or TH. In our analysis of the microarray data, we first determined three basic gene lists, in which genes were significantly differentially expressed in CORT, TH, or CORT-TH treatment compared with control treatment. We then arranged those genes in a Venn diagram to separate the gene lists into seven distinct sections (*a–g*) (Fig. 1). The k-means cluster algorithm was used to analyze each section to cluster genes into hormone-induced expression patterns.

Determine clusters of gene expression patterns

Step 1: altered gene regulation in each treatment vs. control treatment

We found 1968 genes differentially expressed in response to CORT, 3227 genes differentially expressed in response to TH, and 4517 genes differentially expressed in response to CORT-TH. A Venn diagram using these three gene lists populated all seven sections (*a–g*) of the Venn diagram with genes (Fig. 1 and Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Step 2: altered gene regulation requiring the presence of both hormones

Of the 4517 genes in the CORT-TH treatment, 1208 genes (Fig. 1A, section *a*) were expressed differently from control only when both hormones were present. These 1208 represent 22% of total 5432 genes regulated by either one or both hormones. These genes were then clustered with k-means to find genes up (A1) and down (A2) regulated only in the presence of both hormones (Fig. 1B and Table 3). The remaining 3309 genes, in which CORT and/or TH alone was also able to induce significant up- or down-regulation of the genes, are found in other sections (sections *b*, *c*, and *g*) and are analyzed in the next steps.

Step 3: genes differentially regulated in all conditions

A total of 887 genes (Fig. 1A, section *b*) were regulated by each hormone treatment. Using k-means clustering, the list was divided into two clusters B1 and B2, which represent genes up- and down-regulated in each condition against the control treatment (Fig. 1B). Further analysis was required to discern significant differences, if any, in
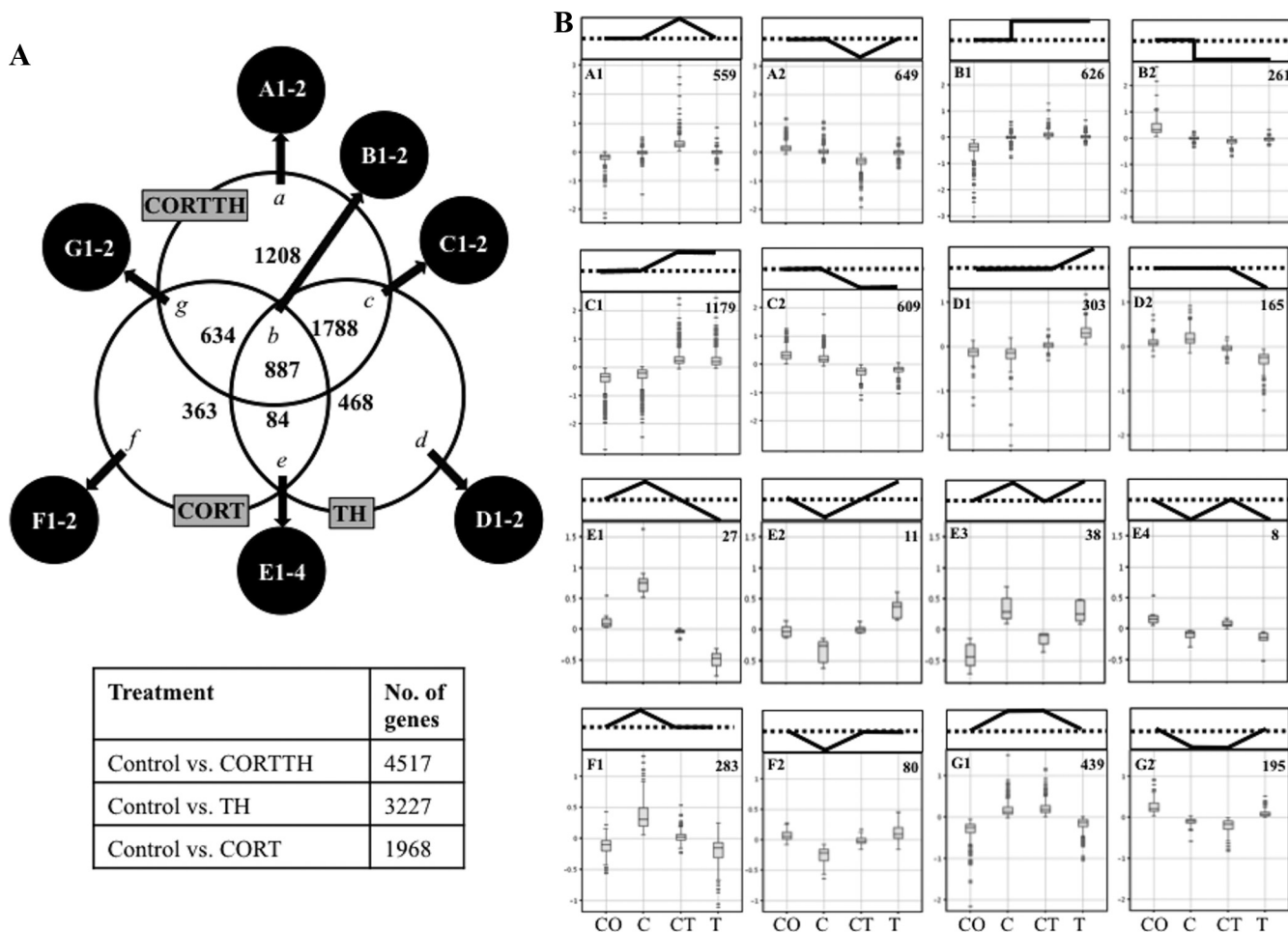
FIG. 1. Number of genes and clusters of gene expression patterns across CORT, TH, and CORT-TH treatments. A, The Venn diagram using the gene lists from CORT, TH, and CORT-TH treatments with the total number of genes indicated in the table resulted in seven different gene regulation sections (*a–g*). B, Each section (*a–g*) from panel A was analyzed using k-means cluster analysis to sort genes into clusters of gene expression patterns (clusters A1–G2). The name of the cluster and number of genes in the cluster are indicated in the *upper corners of the graphs*. Diagrams above each graph indicate the idealized gene expression pattern for each cluster. The y-axis indicates relative expression levels, and the x-axis indicates hormone treatments: CO, Control; C, CORT; CT, CORT-TH; T, TH. Box plots are given for each cluster and hormone treatment.

expression levels for these genes among hormone treatments and to potentially identify additional clusters of gene expression patterns. To this end, we identified genes differentially expressed between CORT-TH and TH and genes differentially expressed between CORT-TH and CORT using GeneSpring (table in Fig. 2). Then we produced a Venn diagram with these two gene lists and genes from section *b* (Fig. 2A). Analysis of this Venn diagram showed that 451 of the 887 genes of section *b* represent no significant expression differences among hormone treatments (but still different from control) (Fig. 2A). These genes exactly match the pattern diagram for clusters B1 and B2 (Fig. 1B). The remaining 436 genes of the 887 in the three remaining sections (Fig. 2A) were then analyzed by k-means to find different patterns of gene regulation. Six patterns of gene expression were found, in which one of the hormone treatments was significantly different from

the other two hormone treatments (Fig. 2, A and D, clusters H1–6).

Step 4: genes differentially regulated in response to TH treatment

A total of 3227 genes were differentially regulated in response to TH compared with control. Of the 3227 genes, 1788 (section *c*) were common to TH and CORT-TH treatment, 468 (section *d*) genes were differentially regulated in response to TH only, and 84 genes (section *e*) were regulated by TH and CORT but not CORT-TH (Fig. 1A). The 1788 genes were then analyzed using k-means clustering to find two clusters C1 and C2 of up- and downregulated genes (Fig. 1B). Of these 1788 genes (section *c*), some of them may be exclusively TH response genes (TH only genes), meaning the expression of these genes is affected only by TH and not influenced by the presence or

TABLE 3.   Most regulated annotated genes (*i.e.* has a gene name) in sections A, C, and G

| Unigene/Affymetrix gene ID	Gene title	Fold change	Regulation
Clusters A1 and A2 (CORTTH synergy genes)			
Str.41880	Thioredoxin-like	14.01	Up
Str.58312	Aminopeptidase N-like	6.16	Up
Str.22009	Aldo-keto reductase family 1, member D1 ($\delta4$–3-ketosteroid-5-β-reductase)	4.77	Up
Str.27172	Nicotinamide N-methyltransferase-like	4.74	Up
Str.15717	PDZ and LIM domain 7 (enigma)	4.70	Up
Str.41889	Poly(U)-specific endoribonuclease-like	4.67	Up
Str.52255	Phosphomannomutase 2	3.77	Up
Str.41312	Matrix metallopeptidase 13 (collagenase 3)	3.62	Up
Str.27994	Calcium binding protein P22	3.51	Up
Str.2462	BCL2/adenovirus E1B 19-kDa interacting protein 3	3.42	Up
Str.20203	Novel protein containing a PX domain	5.45	Down
Str.64743	Bile salt export pump-like, partial	5.06	Down
StrJgi.6225.1.S1_a_at	Interferon-induced very large GTPase 1-like	4.27	Down
Str.51450	Serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	3.40	Down
StrEns.9891.1.S1_a_at	Titin-like	3.18	Down
StrJgi.8717.1.S1_at	Multidrug resistance protein 3-like	3.10	Down
Str.32142	Protein FAM181A	2.92	Down
Str.67562	Versican	2.81	Down
Str.51884	Hypothetical protein LOC100497279	2.70	Down
Str.40243	Olfactomedin-like protein 2A precursor	2.67	Down
Clusters C1 and C2 (TH-only genes)			
Str.34015	Runt-related transcription factor 2 (runx2), mRNA	7.49	Up
Str.49882	Phosphate-regulating neutral endopeptidase-like	7.28	Up
Str.52614	MAX dimerization protein 1	6.78	Up
Str.24304	Arylsulfatase I-like	6.39	Up
Str.53061	Deiodinase, iodothyronine, type 3	6.38	Up
Str.11148	Solute carrier family 16 (monocarboxylic acid transporters), member 12	5.52	Up
Str.27735	Annexin A6 (anxa6), mRNA///annexin A6	5.46	Up
Str.27324	Ras-related protein ras-dva	5.39	Up
Str.47076	Stanniocalcin 2	5.38	Up
Str.30617.3.S1_a_at	Fos-related antigen 2-like	5.05	Up
Str.65102	Tyrosine aminotransferase	3.81	Down
Str.52242	Keratin, type I cytoskeletal 47 kDa	3.25	Down
Str.72934	Dnaj homolog subfamily C member 12-like	2.87	Down
Str.6882	MAD1 mitotic arrest deficient-like 1 (yeast)	2.60	Down
Str.11193	Myogenin (myogenic factor 4)	2.47	Down
Str.7586	Procollagen C-endopeptidase enhancer 2	2.45	Down
StrJgi.5940.1.S1_at	Complement C1q tumor necrosis factor-related protein 3-like	2.40	Down
Str.52900	Reticulocalbin 1, EF-hand calcium binding domain	2.35	Down
Str.54203	RAR-related orphan receptor A	2.32	Down
Str.53703	Butyrobetaine (γ), 2-oxoglutarate dioxygenase (γ-butyrobetaine hydroxylase) 1	2.28	Down
Clusters G1 and G2 (CORT-only genes)			
Str.6047	Phosphoenolpyruvate carboxykinase 1 (soluble)	12.48	Up
Str.6151	ATPase, Na+/K$^+$ transporting, $\beta2$ polypeptide	3.67	Up
StrJgi.2872.1.S1_at	Gastrin-releasing peptide-like	3.17	Up
StrJgi.7877.1.S1_s_at	Putative *N*-acetyltransferase C7orf52-like	3.03	Up
Str.66407	Novel protein similar to X-epilectin	2.70	Up
Str.6235	HIG1 domain family, member 1A	2.54	Up
Str.2174	Anterior gradient 1	2.53	Up
Str.1534.1.S2_a_at	Hypothetical protein LOC100495042	2.45	Up
Str.38128	Predicted: transient receptor potential cation channel subfamily M member 6-like	2.43	Up
Str.21647	Predicted: neurobeachin-like	2.41	Up
Str.53193	Protein FAM113A-like	2.82	Down
Str.37409	Ankyrin repeat and SOCS box-containing 12	2.29	Down
StrEns.4794.1.S1_at	Transmembrane protease, serine 2	1.93	Down
Str.15478	Coiled-coil domain-containing protein 69 mRNA	1.85	Down
Str.66758	Hemicentin-1-like	1.85	Down
StrEns.2846.1.S1_at	Solute carrier family 26, member 7	1.76	Down
StrJgi.5544.1.S1_s_at	Matrix-remodeling-associated protein 5-like	1.67	Down
StrEns.2129.1.S1_at	Protein CBFA2T3-like	1.63	Down
Str.14125	Enah/Vasp-like	1.62	Down
StrJgi.860.1.S1_at	Laminin, $\beta2$ (laminin S)	1.55	Down
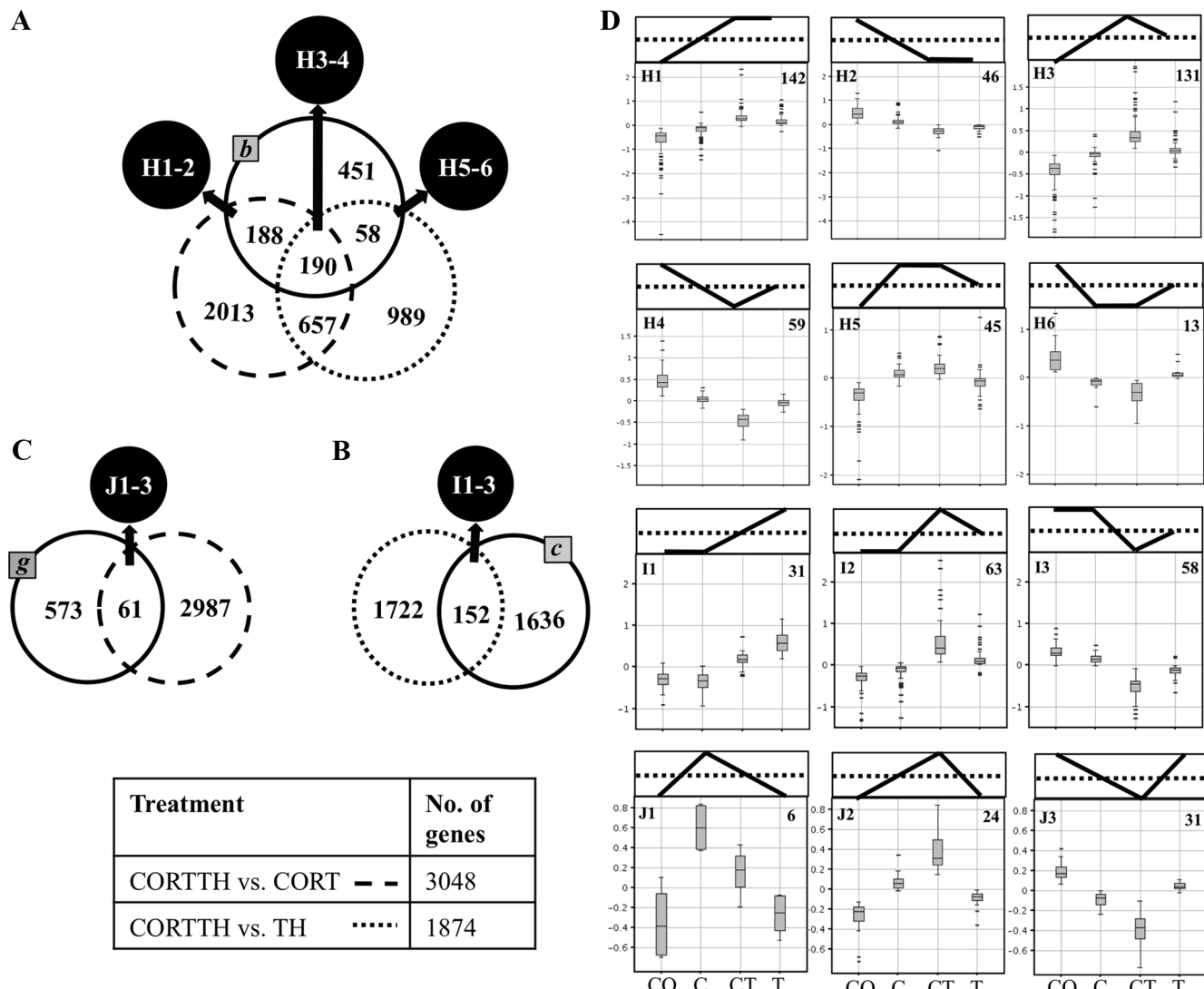
FIG. 2. Subdivision of CORT-TH gene expression patterns and identification of TH-only and CORT-only genes. A, Section *b* (887 genes) from Fig. 1 was analyzed with a Venn diagram using both gene lists in the table, which indicates the number of genes that are significantly different between CORT-TH and CORT or TH treatments. The *dashed lines* in the table match the *dashed lines* in the Venn diagram. Most often (451 genes), the three hormone treatments did not vary in the level of gene regulation, and thus, for these genes their regulation matches the pattern diagramed for clusters B1 and B2. For the other genes, k-means clustering sorted genes into patterns in which one of the three hormone treatments was different from the other two (clusters H1–6) as shown in panel D. B, A Venn diagram with two gene lists, namely genes in section *c* (1788 genes) and genes significantly different between CORT-TH and TH, was used to separate out genes from genes in section *c*, which were significantly different between CORT-TH and TH treatments. Most of the genes (1636) were not significantly different between CORT-TH and TH and therefore were designated as true TH-only genes. The other 152 genes have as yet uncharacterized influence of CORT on their regulation and were sorted into three clusters (I1–3) with k-means clustering as shown in panel D. C, A Venn diagram with two gene lists, namely genes in section *g* (634 genes) and genes significantly different between CORT-TH and CORT, was used to separate out genes from genes in section *g*, which were significantly different between CORT-TH and CORT treatments. Most of the genes (573) were not significantly different between CORT-TH and CORT and therefore were designated as true CORT-only genes. The other 61 genes have as-yet-uncharacterized influence of TH on their regulation and were sorted into three clusters (J1–3) with k-means clustering as shown in panel D. D, Sections from the Venn diagrams in panels A, B, and C were analyzed using k-means cluster analysis to sort genes into clusters of gene expression patterns (clusters H1–J3). The name of the cluster and number of genes in the cluster are indicated in the *upper corners of the graphs*. Diagrams above each graph indicate the idealized gene expression pattern for each cluster. The y-axis indicates relative expression levels, and the x-axis indicates hormone treatments: CO, Control; C, CORT; CT, CORT-TH; T, TH. Box plots are given for each cluster and hormone treatment.

absence of CORT. Thus, the expression levels of TH-only genes should not be significantly different between TH and CORT-TH treatments. To segregate away genes significantly different between TH and CORT-TH, we created a Venn diagram composed of two gene lists, the 1788

genes and those significantly different between TH and CORT-TH treatment (Fig. 2B). We found a total of 152 genes (Fig. 2, B and D, clusters I1–3), which were significantly different between TH and CORT-TH treatments from the list of 1788 genes. The remaining 1636 genes

were designated as TH-only genes (Table 3 and Supplemental Table 1).

The set of 468 genes (section *d*) represents the case in which TH treatment alone altered gene expression. This gene list was then further divided by k-means clustering into clusters D1 and D2, representing up- and down-regulated genes (Fig. 1B). CORT alone cannot induce changes in these genes, but CORT appears to block the action of TH on these genes because they are not regulated in the CORT-TH treatment.

The remaining 84 genes (section *e*) present a special case in which genes are only differentially regulated if one or the other hormone is present but not when both are present together. These genes were divided by k-means clustering into 4 clusters E1–4 (Fig. 1B). E1 represents the case in which CORT in isolation up-regulates the gene, TH in isolation down-regulates the genes, and together they antagonize each other's effect to bring back the gene expression similar to control. E2 represents similar case only with opposite roles of CORT and TH in isolation. E3 and E4 represent special cases because both hormones change gene expression in same direction, either up (E3) or down (E4) when present in isolation but antagonize each other's effect when present together.

Step 5: genes differentially regulated in response to CORT treatment

Of 1968 genes differentially regulated in response to CORT, 363 (section *f*) were differentially regulated only in response to CORT, 634 genes (section *g*) were common to CORT and CORT-TH treatments, and 84 genes (section *e*) were discussed above (Fig. 1A). The 363 genes (section *f*) were divided by k-means clustering into two clusters F1 and F2, representing up- and down-regulated genes (Fig. 1B). These genes (section *f*) represent the case in which CORT alone induced significant up- or down-regulation of a gene, and TH abolished the effect of CORT, even though TH alone did not affect their expression.

Of the 634 genes (section *g*), some of them may be exclusive CORT response genes (CORT-only genes) because the expression of these genes changed only by CORT treatment uninfluenced by the presence or absence of TH. Other genes of the 634 may be differentially expressed between CORT and CORT-TH, indicating an influence by TH. To find CORT-only response genes, we created a Venn diagram composed two gene lists, the 634 genes and those significantly different between CORT and CORT-TH treatment (Fig. 2C). We found a total of 61 genes (Fig. 2, C and D, clusters J1–3), which were significantly different between CORT and CORT-TH treatments from the list of 634 genes. The re-
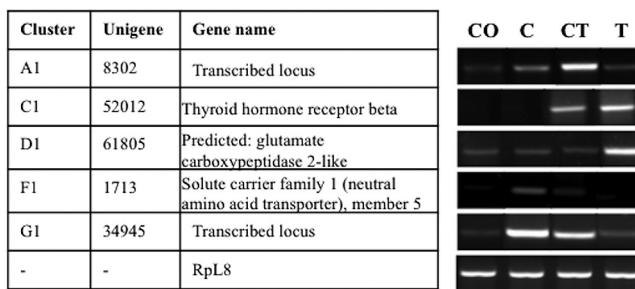

FIG. 3. RT-PCR analysis of selected genes. Five genes, one for each cluster A1, C1, D1, F1, and G1, were analyzed using the same RNA as used in the microarray. *RpL8* was used as a housekeeping gene, and its expression was the same across all samples. The Unigene number and gene description are given. Hormone treatments were abbreviated as follows: CO, Control; C, CORT; CT, CORT-TH; T, TH.

maining 573 genes were designated as CORT-only genes (Table 3 and Supplemental Table 1).

RT-PCR analysis

The gene expression patterns for TRβ and KLF9 obtained from the microarray data analysis were similar to the RT-PCR analysis done on the RNA samples to confirm the effect of TH and CORT, respectively (data not shown). We tested eight genes from five clusters (A1, C1, D1, F1, and G1) with RT-PCR analysis to establish examples of genes exhibiting novel interactions to TH and CORT (Fig. 3).

Meta-analysis for verification of microarray data

We verified our microarray data by comparing our list of TH-responsive genes with those published by previous studies. We found that more than 95% of the genes shown in Table 1 (most highly TH regulated genes in the tail) from Das *et al.* (59) were also significantly up-regulated in our study. Specifically, of 28 up-regulated genes in Table 1 of the report by Das *et al.* (59), six genes were not represented on the Affymetrix *X. tropicalis* microarray, and of the remaining 22 genes, only one gene was not up-regulated in response to TH in our study (Table 2). We also compared our TH-regulated genes with the core set of TH-regulated genes in tail, brain, intestine, and hind limb presented by Buchholz *et al.* (57), which resulted in 71% similarity. Specifically, of the 59 core T_3-response genes in Buchholz *et al.* (57), 11 were not represented in *X. tropicalis* Affymetrix GeneChip. Of the remaining 48 genes, 34 genes ($\sim$71%) were up-regulated by TH, three were down-regulated, and the remaining 12 were unchanged in their expression levels (data not shown). Lastly, we compared our TH results to the union set of three tail array experiments identified by Searcy *et al.* (59). Specifically, out of these 12 TH-response genes, one of the genes (gene 12–1b) was not represented on *X. tropicalis* gene chip. However, all remaining 11 genes (*GAP43,*

MMP2, TIMP2, FN1, MAMDC2, MMP13, FAPα, CREB3, DIO3, ARG2, TBX2 and *gene 17*) were similarly regulated by our TH treatment, which resulted in 100% similarity. Further, some of these genes (*MMP2, FN1, MMP13, FAPα, DIO3, MAMDC2*) were previously verified by PCR by Wang and Brown (1993).

Discussion

CORT is the primary stress hormone in tadpoles and its actions on development are complex and dependent on the presence of TH (37, 38, 40, 41, 43–46, 64, 71). However, the molecular basis of CORT and TH cross talk at the level of gene regulation is still not well understood. Therefore, comprehensive knowledge of genes regulated by CORT and TH during metamorphosis would significantly contribute to our understanding of their actions on gene regulation and metamorphosis. Our study on hormone-treated tails in *X. tropicalis* is the first to find CORT-response genes in tadpoles using microarray analysis, revealing novel interactions between TH and CORT on gene expression. We found that the CORT-TH treatment had the most regulated genes, followed by the TH treatment, and then CORT. We identified genes regulated by CORT alone as well as genes regulated by expected and unexpected interactions with TH.

Verification of microarray data by meta-analysis

In light of the differences in TH dose (100 *vs.* 50 nm) and treatment duration (48 *vs.* 18 h) between Das *et al.* (2006) and our study, the result that more than 95% of the genes shown in the Table 1 of Das *et al.* (2006) (most highly TH-regulated genes in the tail) suggests that the results are highly reproducible. We further show that such high degree of similarity is not confined only to highly regulated genes but also to other TH response genes published by Helbing *et al.* (2003), Das *et al.* (2006) and Searcy *et al.* (64). In addition, identification of 71% of the core TH-response genes among tissues identified by Buchholz *et al.* (57) also indicates a high degree of reproducibility. The remaining 29% (14 of 48 genes) from the core set of genes that were not similarly regulated in our data may reflect the fact that the core set was derived from results from at least 2 d of treatment for each tissue. Because many transcription factors are commonly induced among tissues found in the core gene list (53), it may be that secondary response genes are also represented in the core gene list. Thus, our list from an 18-h treatment enriched in direct-response genes may not contain the putative secondary response genes. Overall, our tail analysis identified many genes in common with previ-

ous tail microarray analyses and indicates reproducibility among microarray experiments.

Clusters of gene expression patterns

We plotted a Venn diagram using genes significantly regulated in CORT, TH, or CORT-TH *vs.* control treatments. Cluster analyses of genes in each of the seven sections of the Venn diagram showed a surprising range of gene expression patterns. Expected patterns included independent regulation of genes by CORT (in section *g*; Fig. 1A) or TH (in section *c*; Fig. 1A) and additive and synergistic interactions on gene regulation between the two hormones (section *a*; Fig. 1A and genes in clusters H3–4; Fig. 2A). A set of unanticipated antagonistic patterns of hormone interaction on gene expression included subtractive, inhibitory, and annihilatory effects. Subtractive interaction was when each hormone in isolation oppositely regulated genes, but when both hormones were present together, they canceled out each other's effect (clusters E1–2; Fig. 1B). Inhibitory interaction was when one of the hormones had no effect on gene expression by itself but only antagonized the effect of the other hormone (clusters D1–2 and F1–2; Fig. 1B). Annilhilatory interaction was when each hormone by itself regulated a gene in the same direction (up or down) as the other hormone, but both hormones together blocked regulation of that gene (clusters E3–4; Fig. 1B). Interestingly, several of these patterns of interaction were observed in a study on TH and CORT regulation of six genes expressed in the hypothalamus selected for their role in energy homeostasis (21). In particular, they found patterns that match cluster B2 (genes *TRH* and proopiomelanocortin), D2 (gene *TRkB*), cluster E4 (gene *LEPR*), cluster F1 (gene *NPY*), and cluster G2 (gene *CRH*).

The various gene expression patterns induced by CORT, TH, and CORT-TH may be explained by the presence of hormone response elements (HRE). Under the assumption that all genes regulated by TH or CORT in our study were direct-response genes, putative HRE can be hypothesized to explain each interaction between TH and CORT (Fig. 4). HRE can be positive or negative resulting in up- or down-regulation of a gene in response to a hormone. Complexities arise when TH response elements (TRE) and GRE regulate the same gene, in which each HRE can be either positive or negative. A further complication is that the regulation of a given gene may not always be direct but could be regulated indirectly by a transcription factor regulated by a TRE or GRE. Without further testing, we cannot determine the mechanisms of CORT and TH interactions at the level of gene regulation, direct *vs.* indirect. However, Das *et al.* listed the 12 direct TH-
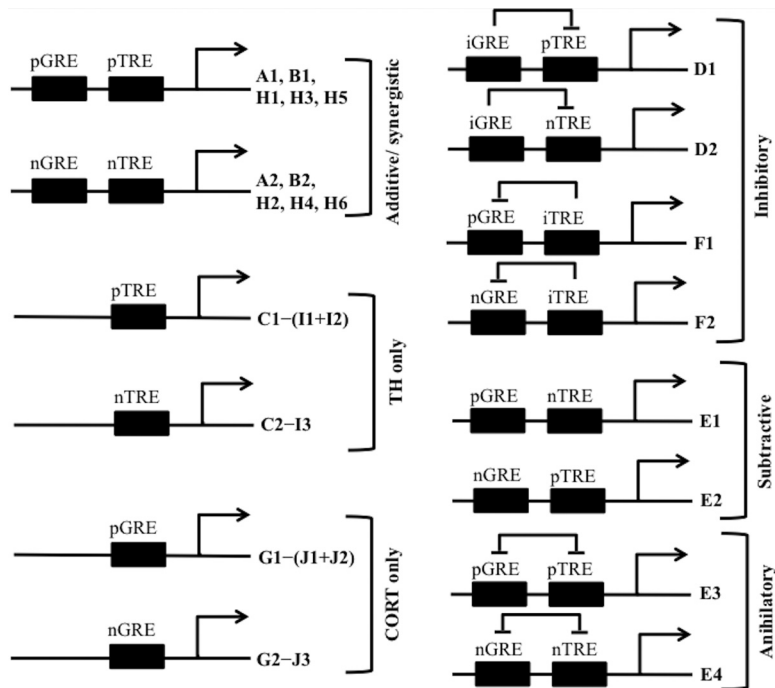
FIG. 4. Predicted hypothetical regulatory interactions between GRE and TRE in different clusters. The predicted interactions are applicable only if both hormones directly regulate the genes. For each diagram of the six regulatory interactions between GRE and TRE, a positive, negative, and/or inhibitory GRE and TRE (pGRE, pTRE, nGRE, nTRE, iGRE, and iTRE) are shown in front of a hypothetical transcriptional start site (*bent arrow*). Associated clusters and terms for the regulatory interaction are given next to each interaction diagram. Note that iGRE and iTRE, as well as the annihilatory interactions, are hypothetical, and the molecular mechanisms underlying such interactions have not thus far been empirically verified.

response genes known to have a TRE in their promoter region [Fig. 6A in Das *et al.* (58)]. Of those 12 genes, we found nine genes (*TH/bZIP*, *TRβ*, *PAAF1*, *CCNJ*, *REV1*, *MMP11*, *MMP13*, and *DIO3*) in cluster C1 and a single gene (*KLF9*) in cluster H1. One of the genes (gene 12–1b) is not present on the *X. tropicalis* chip, and the last remaining gene (*DNase1L3*) did not show any regulation in response to our TH treatment. This analysis was not available for CORT-response genes because of the lack of previous comparable studies. Only in the case of *KLF9* has the mechanism of interaction been demonstrated, namely the promoter of *KLF9* contains both a GRE and a TRE (22, 62). For the following discussion, we note the following: 1) the likelihood that some genes in our study are secondary response genes and 2) the possibility that gene regulation in some clusters, representing novel interactions identified by us, is not mediated by the two hormones *in cis*. Nevertheless, our results reveal novel interactions between CORT and TH, be they direct or indirect.

Section *a* clustered into up- and down-regulated genes (clusters A1 and A2) and represents genes regulated only if both hormones are present together, *i.e.* synergistic regulation in which neither hormone alone is sufficient to alter their expression levels. At face value, these genes ex-

hibit synergistic regulation, with the prediction that these genes possess a positive TRE and GRE or a negative TRE and GRE and that both the thyroid hormone receptor and glucocorticoid receptor complexes must interact with each other to change the expression level of these genes. Other explanations are possible, including one hormone inducing a transcription factor required for direct regulation of the gene by the other hormone. Also, it may be that TH and/or CORT can regulate the genes, but the hormone regulation was not significant in the microarray analysis. However, the TH and CORT levels in the hormone treatments are on the high side, suggesting that even if TH or CORT individually could regulate these genes with higher doses, it may not be biologically significant for the tadpole. Importantly, the clusters from section *a* (clusters A1 and A2) possess the second largest number of genes (second only to clusters in section *c*), emphasizing the importance of the synergistic regulation of these genes during amphibian metamorphosis.

Section *b* clustered into up-regulated in all treatments (B1) and down-regulated in all treatments (B2) and represents genes regulated by both hormones individually and together. Because each hormone, CORT or TH, individually regulated these genes, each of these genes may have both a TRE and a GRE (either both positive or both negative). The presence of a TRE and GRE may allow for additive or synergistic regulation when both hormones are added together, and therefore, further analysis was performed as described in *Results* to determine these hormone interactions (Fig. 2A). Most often (451 genes), the three hormone treatments did not vary significantly in the level of gene regulation. Presumably the regulation of these genes is maximal in the presence of one hormone, such that the presence of both hormones cannot regulate it further. For the other genes in section *b*, this analysis revealed six subclusters (H1–6), in which the gene expression levels were different in one of the three hormone treatments compared with the other two (Fig. 2, A and D). For example, clusters H1 and H2 showed that although CORT significantly affected gene expression, the effect of TH and CORT-TH on gene expression was significantly greater. Similarly, clusters H5 and H6 showed that CORT and CORT-TH had a greater effect than TH alone. Clusters H3 and H4, on the other hand, showed that both TH and CORT had the same effect on gene expression but had a greater effect on gene expression when present together.

Sections *c* and *g* clustered into genes regulated only by TH and CORT-TH (clusters C1 and C2) and only by CORT and CORT-TH (clusters G1 and G2), respectively. For genes in clusters C1 and C2, it is possible that CORT has no influence on these genes, termed TH-only genes. Alternatively, CORT may influence their regulation, but this regulation was not significant in the microarray analysis. Similarly, for genes in clusters G1 and G2, TH may not influence their regulation (CORT-only) or TH may affect them directly, but it was not significant. To better identify TH-only and CORT-only genes, we carried out further analysis as described in *Results* by comparing two gene lists in the Venn diagrams (Fig. 2, B and C). Most genes in section *c* (1636 genes) were thus designated at TH-only, and most genes in section *g* (573 genes) were designated as CORT-only. Assuming that TH-only genes are direct-response genes, they are expected to possess only a TRE, and CORT-only genes are expected to possess only a GRE. The 152 genes partitioned out of section *c* (Fig. 2B) formed three clusters (clusters I1–3), representing TH-response genes with uncharacterized influence of CORT on their regulation. Similarly, the 61 genes partitioned out of section *g* (Fig. 2C) formed three clusters (clusters J1–3), representing CORT-response genes with uncharacterized influence of TH on their regulation.

Sections *d* and *f* represent TH- or CORT-response genes whose up- or down-regulation (clusters D1 and F1 or clusters D2 and F2) can be blocked by the presence of the other hormone. For example, in cluster D1, CORT does not affect the regulation of these genes by itself but antagonizes the gene regulation by TH because the TH induction of the cluster D1 genes is blocked in the cotreatment with CORT (if CORT had no influence, then these genes would also be induced by the CORT-TH treatment). Genes in sections *d* and *f* presumably have a TRE or a GRE, respectively. The mechanism of inhibition by CORT on section *d* genes and TH on section *f* genes is not clear. A novel type of inhibitory HRE may exist in which CORT would bind an inhibitory GRE (iGRE) and act somehow to block TH-induced gene regulation (Fig. 4). Alternatively, the CORT inhibition of these TH-response genes may be indirect via a transcription factor induced by CORT that then inhibits these TH-response genes. In any case, this effect of the two hormones reflects a previously unrecognized inhibitory mode of CORT and TH interaction.

Section *e* represents genes regulated by each hormone individually but not by both hormones together. Clustering analysis revealed four clusters, in which gene regulation by each hormone individually was in the opposite direction (clusters E1 and E2) or in the same direction (clusters E3 and E4), but when both hormones were pres-

ent, the expression level of these genes was not different from the control treatment. Gene regulation in clusters E1 and E2 may be due to a positive GRE and a negative TRE or vice versa such that when both hormones are present, the summation of their individual effects subtract or cancel each other out leading to a net zero effect on gene expression levels. The regulation of cluster E3 and E4 genes is not as easy to interpret. Genes in clusters E3 and E4 appear to have positive GRE and TRE or negative GRE and TRE when CORT or TH alone is considered. However, in the presence of both hormones, gene regulation in clusters E3 and E4 is inhibited. The mechanism of annihilation or mutual antagonism of CORT and TH on regulation of these genes is not clear and represents another novel mode of CORT and TH interaction detected by this study.

Based on the number of genes, the TH-only-regulated clusters (clusters C1 and C2) followed by additive/synergistic clusters (clusters A1, A2, B1, and B2) are the most significant patterns of interaction. The other clusters have many fewer genes. However, comparing the developmental significance of these clusters is difficult due to lack of functional studies on most of the genes such that smaller clusters may have some genes of large effect on development or play specific or vital roles during different stages of development. The following discussion addresses potential functional relevance of these patterns of hormone interaction.

Gene ontology analysis (Table 4)

GO terms associated with genes that are synergistically up-regulated by the CORT-TH treatment (clusters A1 and H3) include intracellular protein transport, vesicle-mediated transport, protein localization, and cellular localization. An example gene is *SLC39A7*, a zinc transporter protein, which is highly up-regulated in response to CORT-TH (72, 73). Zinc is involved in protein, nucleic acid, carbohydrate, and lipid metabolism as well as in the control of gene transcription, growth, development, and differentiation (72–75). Zinc cannot passively diffuse across cell membranes and requires specific transporters, such as SLC39A7, to enter the cytosol from both the extracellular environment and from intracellular storage compartments (72, 73). We found that genes that are down-regulated independently or synergistically by TH and CORT (cluster H4) are associated with negative regulation of cell differentiation or development, consistent with the action of these hormones to promote tail resorption.

Genes up-regulated by CORT (clusters F1 and G1) are associated mainly with metabolic processes and energy production in mitochondria. CORT plays a very impor-

TABLE 4. Clusters with GO terms significantly associated with them

Clusters	Description	GO term	No. of genes
A1	CORT-TH synergy up	Proteasome complex	2
		Cytosol	11
		Intracellular protein transport	8
		Protein localization	17
		Protein transport	17
		Vesicle-mediated transport	9
		Macromolecule localization	17
		Protein complex	13
		Cytoplasmic part	11
		Establishment of protein localization	17
		Cellular localization	8
		Establishment of localization in cell	8
F1	CORT up	Ribosome	7
		RNA processing	2
		Gene expression	2
		RNA metabolic process	3
		Ribonucleoprotein complex	9
		Biopolymer metabolic process	3
G1	CORT-only up	Cytoplasm	25
		Mitochondrion	12
		Mitochondrial envelope	7
		Mitochondrial inner membrane	7
		Transport	17
		Acyltransferase activity	2
		Transferase activity, transferring acyl groups	2
		Organelle inner membrane	7
		Organelle membrane	7
		Mitochondrial membrane	7
		Organelle envelope	7
		Envelope	7
		Mitochondrial part	7
		Cytoplasmic part	12
		Localization	17
		Establishment of localization	17
		Intracellular protein transport	6
		Transferase activity, transferring groups other than amino-acyl groups	2
H1	All regular CORT significantly different up	Endosome	3
		Protein localization	6
		Endosome membrane	1
		Protein transport	6
		Macromolecule localization	6
		Endosomal part	1
		Establishment of protein localization	6
H3	All regular CORT-TH synergy up	Peroxisome fission	2
		Organelle fission	2
H4	All regular CORT-TH synergy down	NAD+ nucleosidase activity	1
		Regulation of cell differentiation	2
		Negative regulation of cell differentiation	2
		Negative regulation of developmental process	2
H6	All regular TH significantly different down	Carboxypeptidase activity	1
		Metallocarboxypeptidase activity	1
		Metalloexopeptidase activity	1

tant role in metabolism, and it makes sense that an increase in CORT would increase the expression of genes like phosphoenolpyruvate carboxykinase, serine dehydratase, and ethylmalonic encephalopathy 1. These genes play important roles in gluconeogenesis (76–80). In fact, research in mammals has shown that mutations in the ethylmalonic encephalopathy 1 gene impair the body's ability to produce energy in mitochondria (79, 80). Tadpoles, during metamorphosis, reduce their feeding drastically and depend on stored energy stores for their energy requirements. CORT may up-regulate these gluconeogenic genes to provide sufficient glucose to the body and brain during

metamorphosis. Interestingly, we have also shown that tadpoles increase the rate of metabolism during stress-induced accelerated metamorphosis (Kulkarni, S.S., D.R. Buchholz, and I. Gomez-Mestre, unpublished data), and the rate of developmental acceleration is associated with a proportional decrease in fat levels (81). Thus, CORT may regulate energy requirements of the body by inducing genes important for altered metabolism appropriate during stress.

GO analysis did not identify significant GO categories for genes significantly regulated by TH; however, when we applied GO analysis on genes significantly and 2-fold up-regulated by TH, we found several familiar significant GO categories, including apoptosis, programmed cell death, amino acid transport, organic acid transport, carboxyl acid transport, and metallopeptidase activity (24, 53, 55, 59).

Biological significance of the microarray data

The diversity of TH/CORT interactions we identified on gene regulation is surprising given the single effect of TH and CORT, *i.e.* synergy, on morphological change during metamorphosis (38–40). Here we discuss how the genes in different patterns of TH and CORT interaction may be involved in regulating development. The large fraction of genes representing CORT and TH synergy (22.5%, clusters A1, A2, B1, and B2) emphasizes the importance of studying the mechanistic basis of amphibian metamorphosis by taking into account the action of these two hormones together, rather than studying the role of these hormones independently in metamorphosis. Although CORT may merely enhance TH signaling by CORT's actions on deiodinase activity and inducing TRβ expression (38, 40, 62), our results suggest additional significant interactions between CORT and TH as discussed below.

We propose that clusters F1 and F2 represent genes important in the stress response early in life, which may inhibit premetamorphic tadpole development. In these clusters, genes are up- and down-regulated by CORT in the absence of TH but in the presence of TH, expression of these genes returns to control levels. During early tadpole development, increased CORT in response to a stressor may significantly activate these genes and, in the absence of TH, may have the effect of delaying development. However, during prometamorphosis, the presence of TH promotes tadpole development, perhaps in part by inhibiting CORT regulation of these genes. Thus, clusters F1 and F2 may represent a set of genes important in uncovering the mechanistic basis of the dual role of CORT in amphibian metamorphosis.

The metabolic function of CORT-regulated genes may also provide some insight into why the increase in CORT during premetamorphosis retards development. Tadpoles cannot initiate metamorphosis before a certain size is reached, and it is hypothesized that some endocrine signal indicates availability of enough energy resources to initiate metamorphosis (82, 83). However, increase in CORT in response to a stressor during premetamorphosis may increase gluconeogenesis and use stored energy resources to respond to a stressor. This may delay the signal that indicates availability of resources to initiate metamorphosis and thus CORT may retard initiation of metamorphosis.

Later in life, prometamorphic tadpoles are well known for their ability to accelerate metamorphosis in response to stressors like desiccation (developmental plasticity), a response mediated by CORT (23, 81, 84). Acceleration of development during metamorphosis needs both hormones (84–86), and our data contribute to how the two hormones may work together. In the absence of TH, there is no metamorphic initiation. When TH is present, the acceleratory effect of CORT on development during prometamorphosis may be understood by elucidating the roles of genes regulated by CORT (clusters F1, F2, G1, and G2) *vs.* CORT and TH (clusters A1, A2, B1, and B2) during this period. Because a large fraction of the genes are regulated only in the presence of both hormones, acceleration of development can be inefficient in the presence of only one hormone. Future functional studies are required to unravel the importance of these genes in developmental plasticity.

Although TH is required to initiate metamorphosis, it cannot complete metamorphosis in the absence of CORT (29, 32–35). In particular, tail and gills do not undergo complete resorption in the absence of CORT (34). Therefore, we propose that clusters D1 and D2, representing genes activated by TH whose regulation is inhibited by CORT, may help explain why TH alone is not sufficient to complete metamorphosis, Specifically, blocked T_3-regulation of the genes in clusters D1 and D2 by CORT in the final phases of metamorphosis may be necessary to complete metamorphosis. These genes can be further evaluated to test for such a potential role.

Acknowledgments

References

1. Sapolsky RM, Romero LM, Munck AU 2000 How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21:55–89

2. Wada H 2008 Glucocorticoids: mediators of vertebrate ontogenetic transitions. Gen Comp Endocrinol 156:441–453

3. Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K, Schutz G 1995 Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev 9:1608–1621

4. Grier DG, Halliday HL 2004 Effects of glucocorticoids on fetal and neonatal lung development. Treat Resp Med 3:295–306

5. Kovar J, Waddell BJ, Sly PD, Willet KE 2001 Glucocorticoids and lung development in the fetus and preterm infant. Pediatr Pulmonol 32:8–13

6. Liggins GC 1994 The role of cortisol in preparing the fetus for birth. Reproduction, Fertil Dev 6:141–150

7. Fowden AL, Li J, Forhead AJ 1998 Glucocorticoids and the preparation for life after birth: are there long-term consequences of the life insurance? Proc Nutr Soc 57:113–122

8. McCormick CM, Smythe JW, Sharma S, Meaney MJ 1995 Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. Brain Res Dev Brain Res 84:55–61

9. Young JB, Morrison SF 1998 Effects of fetal and neonatal environment on sympathetic nervous system development. Diabetes Care 21:B156–B160

10. Pohlová I, Mikšík I, Pácha J 1997 The role of 11β-hydroxysteroid dehydrogenase in maturation of the intestine. Mech Ageing Dev 98:139–150

11. Schmidt M, Sangild PT, Blum JW, Andersen JB, Greve T 2004 Combined ACTH and glucocorticoid treatment improves survival and organ maturation in premature newborn calves. Theriogenology 61:1729–1744

12. Sangild PT, Hilsted L, Nexo E, Fowden AL, Silver M 1994 Secretion of acid, gastrin, and cobalamin-binding proteins by the fetal pig stomach: developmental regulation by cortisol. Exp Physiol 79:135–146

13. Sangild PT, Weström BR, Silver M, Fowden AL 1995 Maturational effects of cortisol on the exocrine abomasum and pancreas in fetal sheep. Reprod Fertil Dev 7:655–658

14. Schreck CB 1993 Glucocorticoids: metabolism, growth, and development. In Schreibman MP, Scanes CG, Kai P, Pang T, eds. The endocrinology of growth, development, and metabolism in vertebrates. Waltham, MA: Academic Press; 367–392

15. Sangild PT, Fowden AL, Trahair JF 2000 How does the foetal gastrointestinal tract develop in preparation for enteral nutrition after birth? Livest Prod Sci 66:141–150

16. Myatt L, Sun K 2010 Role of fetal membranes in signaling of fetal maturation and parturition. Int J Dev Biol 54:545–553

17. Kulkarni SS, Singamsetty S, Buchholz DR 2010 Corticotropin-releasing factor regulates the development in the direct developing frog, Eleutherodactylus coqui. Gen Comp Endocrinol 169:225–230

18. Zhang J, Lazar MA 2000 The mechanism of action of thyroid hormones. Annu Rev Physiol 62:439–466

19. Lazar MA 1993 Thyroid hormone receptors: multiple forms, multiple possibilities. Endocr Rev 14:184–193

20. Yeh KY, Moog F 1975 Development of the small intestine in the hypophysectomized rat. II. Influence of cortisone, thyroxine, growth hormone, and prolactin. Dev Biol 47:173–184

21. Byerly MS, Simon J, Lebihan-Duval E, Duclos MJ, Cogburn LA, Porter TE 2009 Effects of BDNF, T$_3$, and corticosterone on expression of the hypothalamic obesity gene network in vivo and in vitro. Am J Physiol Regul Integr Comp Physiol 296:R1180–R1189

22. Bagamasbad P, Howdeshell KL, Sachs LM, Demeneix BA, Denver RJ 2008 A role for basic transcription element-binding protein 1 (BTEB1) in the autoinduction of thyroid hormone receptor β. J Biol Chem 283:2275–2285

23. Denver RJ 2009 Stress hormones mediate environment-genotype interactions during amphibian development. Gen Comp Endocrinol 164:20–31

24. Shi Y-B 1999 Amphibian metamorphosis: from morphology to molecular biology. New York: Wiley; 1–88

25. Crespi EJ, Denver RJ 2005 Ancient origins of human developmental plasticity. Am J Hum Biol 17:44–54

26. McLean M, Smith R 2001 Corticotrophin-releasing hormone and human parturition. Reproduction 121:493–501

27. Amiel-Tison C, Cabrol D, Denver R, Jarreau PH, Papiernik E, Piazza PV 2004 Fetal adaptation to stress: part II. Evolutionary aspects; stress-induced hippocampal damage; long-term effects on behavior; consequences on adult health. Early Hum Dev 78:81–94

28. Amiel-Tison C, Cabrol D, Denver R, Jarreau H-, Papiernik E, Piazza PV 2004 Fetal adaptation to stress. Part I: acceleration of fetal maturation and earlier birth triggered by placental insufficiency in humans. Early Hum Dev 78:15–27

29. Gudernatsch JF 1912 Feeding experiments on tadpoles—I. The influence of specific organs given as food on growth and differentiation. A contribution to the knowledge of organs with internal secretion. Arch Entwickl Org 35:457–483

30. A BM 1916 The results of extirpation of the anterior lobe of the hypophysis and of the thyroid of Rana pipiens larvae. Science 44:755–758

31. Allen BM 1925 The effects of extirpation of the thyroid and pituitary glands upon the limb development of anurans. J Exp Zool 42:13–30

32. D'Angelo SA, Gordon AS, Charipper HA 1941 The role of the thyroid and pituitary glands in the anomalous effect of inanition on amphibian metamorphosis. J Exp Zool 87:259–277

33. Brown DD, Cai L 2007 Amphibian metamorphosis. Dev Biol 306:20–33

34. Rémy C, Bounhiol JJ 1971 Normalized metamorphosis achieved by adrenocorticotropic hormone in hypophysectomized and thyroxined Alytes tadpoles. Comptes rendus hebdomadaires des seances de l'Academie des Sciences. Serie D Sci Nat 272:455–458

35. Furlow JD, Neff ES 2006 A developmental switch induced by thyroid hormone: Xenopus laevis metamorphosis. Trends Endocrinol Metab 17:40–47

36. Denver RJ 2009 Endocrinology of complex life cycles: amphibians. In Pfaff D, Arnold A, Etgen A, Fahrbach S, Moss R, Rubin R, eds. Hormones, brain and behavior. San Diego: Elsevier; 707–744

37. Galton VA 1990 Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone. Endocrinology 127:2997–3002

38. Bonett RM, Hoopfer ED, Denver RJ 2010 Molecular mechanisms of corticosteroid synergy with thyroid hormone during tadpole metamorphosis. Gen Comp Endocrinol 168:209–219

39. Frieden E, Naile B 1955 Biochemistry of amphibian metamorphosis: I. Enhancement of induced metamorphosis by glucocorticoids. Science 121:37–39

40. Hayes TB 1995 Interdependence of corticosterone and thyroid hormones in larval toads (Bufo boreas). I. Thyroid hormone-dependent and independent effects of corticosterone on growth and development. J Exp Zool 271:95–102

41. Hayes TB, Wu TH 1995 Interdependence of corticosterone and thyroid hormones in toad larvae (Bufo boreas). II. Regulation of corticosterone and thyroid hormones. J Exp Zool 271:103–111

42. **Kikuyama S, Niki K, Mayumi M, Shibayama R, Nishikawa M, Shintake N** 1983 Studies on corticoid action on the toad tadpole tail *in vitro*. Gen Comp Endocrinol 52:395–399

43. **Kikuyama S, Niki K, Mayumi M, Kawamura K** 1982 Retardation of thyroxine-induced metamorphosis by amphenone B in toad tadpoles. Endocrinol Jpn 29:659–662

44. **Glennemeier KA, Denver RJ** 2002 Small changes in whole-body corticosterone content affect larval *Rana pipiens* fitness components. Gen Comp Endocrinol 127:16–25

45. **Wright ML, Cykowski LJ, Lundrigan L, Hemond KL, Kochan DM, Faszewski EE, Anuszewski CM** 1994 Anterior pituitary and adrenal cortical hormones accelerate or inhibit tadpole hindlimb growth and development depending on stage of spontaneous development or thyroxine concentration in induced metamorphosis. J Exp Zool 270:175–188

46. **Darras VM, Van der Geyten S, Cox C, Segers IB, De Groef B, Kühn ER** 2002 Effects of dexamethasone treatment on iodothyronine deiodinase activities and on metamorphosis-related morphological changes in the axolotl (*Ambystoma mexicanum*). Gen Comp Endocrinol 127:157–164

47. **Bonett RM, Hu F, Bagamasbad P, Denver RJ** 2009 Stressor and glucocorticoid-dependent induction of the immediate early gene krüppel-like factor 9: implications for neural development and plasticity. Endocrinology 150:1757–1765

48. **Kikuyama S, Kawamura K, Tanaka S, Yamamoto K** 1993 Aspects of amphibian metamorphosis: hormonal control. Int Rev Cytol 145:105–148

49. **Hollar AR, Choi J, Grimm AT, Buchholz DR** 2011 Higher thyroid hormone receptor expression correlates with short larval periods in spadefoot toads and increases metamorphic rate. Gen Comp Endocrinol 173:190–198

50. **Nakajima K, Fujimoto K, Yaoita Y** 2012 Regulation of thyroid hormone sensitivity by differential expression of the thyroid hormone receptor during *Xenopus* metamorphosis. Genes Cells 17:645–659

51. **Buchholz DR, Paul BD, Fu L, Shi YB** 2006 Molecular and developmental analyses of thyroid hormone receptor function in *Xenopus laevis*, the African clawed frog. Gen Comp Endocrinol 145:1–19

52. **Wang Z, Brown DD** 1993 Thyroid hormone-induced gene expression program for amphibian tail resorption. J Biol Chem 268:16270–16278

53. **Denver RJ, Pavgi S, Shi YB** 1997 Thyroid hormone-dependent gene expression program for *Xenopus* neural development. J Biol Chem 272:8179–8188

54. **Shi YB, Brown DD** 1993 The earliest changes in gene expression in tadpole intestine induced by thyroid hormone. J Biol Chem 268:20312–20317

55. **Buckbinder L, Brown DD** 1992 Thyroid hormone-induced gene expression changes in the developing frog limb. J Biol Chem 267:25786–25791

56. **Brown DD, Cai L, Das B, Marsh-Armstrong N, Schreiber AM, Juste R** 2005 Thyroid hormone controls multiple independent programs required for limb development in *Xenopus laevis* metamorphosis. Proc Natl Acad Sci USA 102:12455–12458

57. **Buchholz DR, Heimeier RA, Das B, Washington T, Shi YB** 2007 Pairing morphology with gene expression in thyroid hormone-induced intestinal remodeling and identification of a core set of TH-induced genes across tadpole tissues. Dev Biol 303:576–590

58. **Das B, Heimeier RA, Buchholz DR, Shi YB** 2009 Identification of direct thyroid hormone response genes reveals the earliest gene regulation programs during frog metamorphosis. J Biol Chem 284:34167–34178

59. **Das B, Cai L, Carter MG, Piao YL, Sharov AA, Ko MS, Brown DD** 2006 Gene expression changes at metamorphosis induced by thyroid hormone in *Xenopus laevis* tadpoles. Dev Biol 291:342–355

60. **Heimeier RA, Das B, Buchholz DR, Fiorentino M, Shi YB** 2010 Studies on *Xenopus laevis* intestine reveal biological pathways underlying vertebrate gut adaptation from embryo to adult. Genome Biol 11:R55

61. **Suzuki K, MacHiyama F, Nishino S, Watanabe Y, Kashiwagi K, Kashiwagi A, Yoshizato K** 2009 Molecular features of thyroid hormone-regulated skin remodeling in *Xenopus laevis* during metamorphosis. Dev Growth Differ 51:411–427

62. **Helbing CC, Werry K, Crump D, Domanski D, Veldhoen N, Bailey CM** 2003 Expression profiles of novel thyroid hormone-responsive genes and proteins in the tail of *Xenopus laevis* tadpoles undergoing precocious metamorphosis. Mol Endocrinol 17:1395–1409

63. **Veldhoen N, Crump D, Werry K, Helbing CC** 2002 Distinctive gene profiles occur at key points during natural metamorphosis in the *Xenopus laevis* tadpole tail. Dev Dyn 225:457–468

64. **Searcy BT, Beckstrom-Sternberg SM, Beckstrom-Sternberg JS, Stafford P, Schwendiman AL, Soto-Pena J, Owen MC, Ramirez C, Phillips J, Veldhoen N, Helbing CC, Propper CR** 2012 Thyroid hormone-dependent development in *Xenopus laevis*: A sensitive screen of thyroid hormone signaling disruption by municipal wastewater treatment plant effluent. Gen Comp Endocrinol 176:481–492

65. **Hasebe T, Kajita M, Fu L, Shi YB, Ishizuya-Oka A** 2012 Thyroid hormone-induced sonic hedgehog signal up-regulates its own pathway in a paracrine manner in the *Xenopus laevis* intestine during metamorphosis. Dev Dyn 241:403–414

66. **Ishizuya-Oka A** 2011 Amphibian organ remodeling during metamorphosis: insight into thyroid hormone-induced apoptosis. Dev Growth Differ 53:202–212

67. **Nieuwkoop PD, Faber J** 1994 Normal table of *Xenopus laevis* (Daudin). New York: Garland Publishing Inc

68. **Helbing CC, Ji L, Bailey CM, Veldhoen N, Zhang F, Holcombe GW, Kosian PA, Tietge J, Korte JJ, Degitz SJ** 2007 Identification of gene expression indicators for thyroid axis disruption in a *Xenopus laevis* metamorphosis screening assay. Part 2. Effects on the tail and hindlimb. Aquat Toxicol 82:215–226

69. **Helbing CC, Bailey CM, Ji L, Gunderson MP, Zhang F, Veldhoen N, Skirrow RC, Mu R, Lesperance M, Holcombe GW, Kosian PA, Tietge J, Korte JJ, Degitz SJ** 2007 Identification of gene expression indicators for thyroid axis disruption in a *Xenopus laevis* metamorphosis screening assay. Part 1. Effects on the brain. Aquat Toxicol 82:227–241

70. **Glennemeier KA, Denver RJ** 2002 Developmental changes in interrenal responsiveness in anuran amphibians. Integr Comp Biol 42:565–573

71. **Suzuki MR, Kikuyama S** 1983 Corticoids augment nuclear binding capacity for triiodothyronine in bullfrog tadpole tail fins. Gen Comp Endocrinol 52:272–278

72. **Eide DJ** 2006 Zinc transporters and the cellular trafficking of zinc. Biochim Biophys Acta 1763:711–722

73. **Huang L, Kirschke CP, Zhang Y, Yu YY** 2005 The ZIP7 gene (Slc39a7) encodes a zinc transporter involved in zinc homeostasis of the Golgi apparatus. J Biol Chem 280:15456–15463

74. **Bitanihirwe BK, Cunningham MG** 2009 Zinc: the brain's dark horse. Synapse 63:1029–1049

75. **King JC** 2011 Zinc: An essential but elusive nutrient. Am J Clin Nutr 94:679S–684S

76. **Leegood RC, ap Rees T** 1978 Phosphoenolpyruvate carboxykinase and gluconeogenesis in cotyledons of *Cucurbita pepo*. Biochim Biophys Acta 524:207–218

77. **Söling HD, Kleineke J, Willms B, Janson G, Kuhn A** 1973 Relationship between intracellular distribution of phosphoenolpyruvate carboxykinase, regulation of gluconeogenesis, and energy cost of glucose formation. Eur J Biochem 37:233–243

78. **Sandoval IV, Sols A** 1974 Gluconeogenesis from serine by the serine dehydratase dependent pathway in rat liver. Eur J Biochem 43:609–616

79. **Tiranti V, D'Adamo P, Briem E, Ferrari G, Mineri R, Lamantea E, Mandel H, Balestri P, Garcia-Silva MT, Vollmer B, Rinaldo P, Hahn SH, Leonard J, Rahman S, Dionisi-Vici C, Garavaglia B, Gasparini**

P, Zeviani M 2004 Ethylmalonic encephalopathy is caused by mutations in ETHE1, a gene encoding a mitochondrial matrix protein. Am J Hum Genet 74:239–252

80. **Wajner M, Goodman SI** 2011 Disruption of mitochondrial homeostasis in organic acidurias: Insights from human and animal studies. J Bioenerg Biomembr 43:31–38

81. **Kulkarni SS, Gomez-Mestre I, Moskalik CL, Storz BL, Buchholz DR** 2011 Evolutionary reduction of developmental plasticity in desert spadefoot toads. J Evol Biol 24:2445–2455

82. **Wilbur HM, Collins JP** 1973 Ecological aspects of amphibian metamorphosis. Science 182:1305–1314

83. **Rose CS** 2005 Integrating ecology and developmental biology to explain the timing of frog metamorphosis. Trends Ecol Evol 20:129–135

84. **Denver RJ** 1997 Environmental stress as a developmental cue: Corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Horm Behav 31:169–179

85. **Denver RJ** 1993 Acceleration of anuran amphibian metamorphosis by corticotropin-releasing hormone-like peptides. Gen Comp Endocrinol 91:38–51

86. **Denver RJ** 1998 Hormonal correlates of environmentally induced metamorphosis in the Western spadefoot toad, *Scaphiopus hammondii*. Gen Comp Endocrinol 110:326–336