Thyroid Hormone Metabolism and Peroxidase Function in Two Non-chordate Animals

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ABSTRACT In mammals, thyroid hormone (TH) signaling is essential for metabolic control, differentiation and homeostasis. These hormones are also involved in the regulation of metamorphosis in amphibians and lampreys and a role in basal chordates has been suggested. Increasing evidence supports TH-related function not only in basal chordates such as urochordates and cephalochordates but also in other invertebrate groups. However, the regulatory mechanisms underlying TH function including the mechanisms of endogenous synthesis of hormones in these groups are essentially unknown. Our data provide evidence for endogenous TH synthesis in the sea hare *Aplysia californica* and the sea urchin *Lytechinus variegatus* based on thin layer chromatography. Pharmacological experiments show that these hormones accelerate development to metamorphosis and specifically affect the formation of juvenile skeletal structures in the sea urchin. Furthermore, we identified two new peroxidase genes (LvTPO from *L. variegatus* and AcaTPO from *A. californica*) showing high sequence similarity with peroxidasin and thyroid peroxidases (the critical TH synthesis enzymes found in all vertebrates). Spatial and temporal expression patterns of these transcripts suggest a role of LvTPO and AcaTPO in a variety of processes such as development to metamorphosis and the regulation of the animal's energetics. We discuss our new findings in the context of evolution of TH synthesis and TH signaling in non-chordate animals. *J. Exp. Zool. (Mol. Dev. Eiol.) 306B, 2006.*

The importance of thyroid hormone (TH; please note that we use the term TH for invertebrates as an abbreviation for thyroid hormone-like compounds throughout because these hormones still await identification using mass spectrometry and other analytical, non-antibody-based methods) signaling for diverse processes such as cell differentiation, growth and metabolism has been well-documented for all vertebrate species investigated to date (*reviewed in* McNabb, '92; Yen, 2001). One critical component of the TH signaling pathway is the synthesis of the hormone. The vertebrate thyroid is a highly specialized organ that is able to concentrate the limited iodine from the environment (Venturi et al., 2000; Truesdale and Bailey, 2002). Subsequently, tyrosine residues present on a thyroglobulin scaffold are iodinated by a series of reactions catalyzed by thyroid peroxidase (TPO) (McLachlan and Rapoport, '92; Taurog, 2000).

The endostyle of basal chordates, a pharyngeal organ involved in filter feeding, is generally accepted as the thyroid homolog and evolutionary predecessor of thyroid glands found in vertebrates (Ogasawara et al., '99; Ruppert et al., '99; Ogasawara, 2000). As a confirmation of this

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Abbreviations: Explanation TH, thyroid hormone; TPO, thyroid peroxidase; MFSW, millipore-filtered seawater; 0.2 μm; T4, thyroxine; SW125 MFSW with I125; T3 (3,3″, 5-triiodo-L-thyronine); TR, thyroid hormone receptor; SULT, sulfotransferase.

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hypothesis, recent investigations revealed the presence of this enzyme in the endostyle of three basal chordates, the ascidians *Ciona intestinalis* and *Halocynthia roretzi* (Ogasawara et al., '99) and the cephalochordate *Brachiostoma belcheri* (Ogasawara, 2000). However, no conclusive evidence on endogenous TH synthesis outside of the chordate clade exists. Moreover, there is little information about the presence of TPO-related genes outside the chordates. Here we provide evidence that endogenous TH synthesis evolved within the echinoderms and within the lophotrochozoans suggesting that this character is not a chordate synapomorphy. Additionally, we propose a new model of TH synthesis via TPO-related peroxidases from the animal peroxidase superfamily.

Several critical components of TH signaling have been investigated in a variety of metazoan taxa except ctenophores, nematodes and echiurans (Fig. 1, *all data compiled from* Eales, '97). For example, several insect species have been shown to incorporate iodine into THs (*reviewed in* Eales, '97). In the polychaete *Eisenia foeta*, radioiodine is incorporated into the central nervous system (CNS) specifically between neurosecretory cells and the neuropile fibers (Davoli et al., '91). Moreover, thyroglobulin-like immunoreactivity was detected in the nervous system of this species (Marcheggiano et al., '85). The gorgonian coral *Leptogorgia irgulata* contains a thyroxine (T4)-like substance which appears to be involved in Ca^{2+} metabolism (Kingsley et al., 2001). Another cnidarian, the jellyfish *Aurelia*, responds to THs by strobilation and it has been further suggested that this cnidarian can synthesize TH precursors endogenously (Spangenberg, '67, '71, '74). More examples are reviewed in Eales ('97) and summarized in Figure 1.

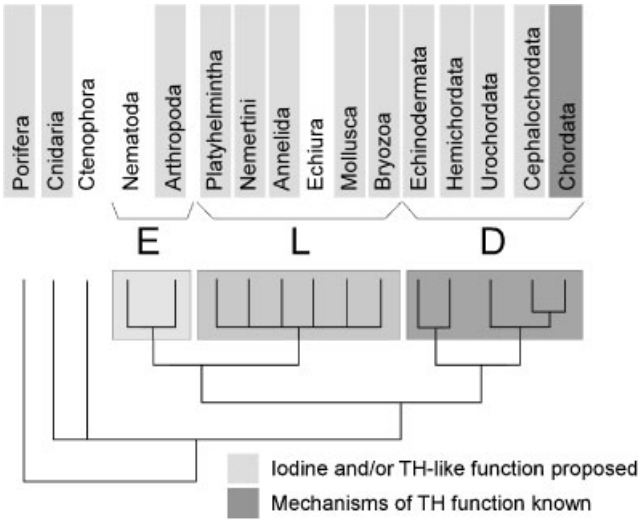

Fig. 1. Thyroid hormone-like function has been proposed for a variety of metazoan phyla but its mechanisms are poorly investigated in the majority of taxa except chordates. We identified two peroxidases potentially involved in thyroid hormone synthesis from the opisthobranch mollusc *Aplysia californica* (AcaTPO) and the echinoderm *Lytechinus variegatus* (LvTPO). Cladogram shows metazoan phylogeny (modified from Peterson and Eernisse, 2001): Except Ctenophores, Nematodes and Echiurans, all metazoan taxa appear to have Iodine and/or TH-like functions (data compiled from Eales et al., 1997 and Heyland et al., 2005). E: Ecdysozoa; L: Lophotrochozoa; D: Deuterostomata; The presence of TH and mechanistic basis of TH action however is only known in chordates (dark shading).

In several echinoids and one sea star, exogenous T4 (one TH) accelerates larval development (Chino et al., '94; Johnson and Cartwright, '96; Hodin et al., 2001; Heyland and Hodin, 2004) and leads subsequently to an earlier metamorphosis (Heyland and Hodin, 2004). The source of endogenous hormone however remains controversial. Several authors have hypothesized that TH or TH-like compounds may primarily originate from unicellular algae in feeding sea urchin larvae (larval type in echinoderms that needs to feed on phytoplankton in order to reach metamorphosis; Chino et al., '94; Heyland and Hodin, 2004; Heyland and Moroz, 2005; Heyland et al., 2005). In contrast, we provided preliminary evidence that feeding larvae of two sand dollar species, *Dendraster excentricus* and *Clypeaster rosaceus* may synthesize thyroid hormone-like compounds endogenously, additionally to the exogenous source (Heyland and Hodin, 2004; Heyland et al., Personal Communication). Moreover, Saito et al. ('98) showed that the lecithotrophic Japanese sand dollar species *Peronella japonica* contains small but detectable amounts of T4 and T3. In all of these studies, the vertebrate goitrogen thiourea inhibited metamorphosis, suggesting the involvement of TPO-related enzymes in thyroid hormone synthesis in non-chordate deuterostomes as well.

Iodine incorporation and the ability to metabolize exogenous THs have been demonstrated for some bivalve and gastropod Molluscs (Antheunisse and Lever, '56; Tong and Chaikoff, '61) but endogenous hormone synthesis and TH-related signaling has not further been investigated in this and other lineages of lophotrochozoan animals. Here, we present evidence for TH-related function in the sea urchin *Lytechinus variegatus* and the marine opisthobranch Mollusc *Aplysia californica*. We show that both *Aplysia* and *Lytechinus* contain THs (T4 and T3) and provide evidence that

these hormones are synthesized from incorporated iodine using thin layer chromatography (TLC). Moreover, we show that T4 accelerates spicule formation and development to metamorphosis in the sea urchin. Finally, we partially sequenced two new peroxidase genes from *Aplysia* and *Lytechinus* that are closely related to peroxidasin of human (PERDSN: HUMAN; Genbank accession number BAA13219), *Drosophila* (PERDSN:DROME, Genbank accession number S46224) and *C. elegans* (PERDSN1:CELE, Genbank accession number CAA91994) and fall into a sister clade to TPOs. The gene product of PERDSN:DROME has been previously shown to synthesize THs in vitro (Nelson et al., '94). Moreover, the expression patterns of AcaTPO and LvTPO suggest roles in development and metabolism in larvae and adults. Their functional involvement in endogenous TH synthesis in Molluscs and echinoderms however remains to be elucidated.

MATERIALS AND METHODS

Animal maintenance and larval culturing

For testing TH and TH metabolite effects on sea urchin development and metamorphosis, we collected adult *L. variegatus* at Jupiter inlet Florida (26°57′28.41″N; 80°04′40.03″W) in October and November 2002 at low tide and from the Keys marine Laboratory on Long Key, Florida in February and October 2003. Upon collection, animals were maintained in the laboratory at 21–24°C in flow-through seawater. We received juvenile and adult *A. californica* from the experimental hatchery in Miami (The University of Miami Experimental Hatchery of the Rosenstiel School of Marine and Atmospheric Science).

To obtain sea urchin larval cultures, we induced spawning by injection of 1 ml 0.55 M KCl solution in the gonad of adult urchins. Eggs were collected in millipore-filtered seawater (MFSW; 0.2 μm) and sperm were collected dry. After eggs had settled, excess water was replaced once with fresh MFSW and a 1:10,000 solution of concentrated sperm was added. About 1 min later, eggs were viewed under the compound microscope to check for fertilization envelopes. Fertilization success was estimated by counting the number of successfully fertilized eggs out of 50 randomly sampled eggs. We only considered the fertilization as successful if fertilization success was more than 95% (successfully fertilized/50). Larvae were maintained in gallon jars filled with 3.8 l MFSW at a concentration of 1 larva/5 ml MFSW. Hatching occurred within 12 hr after fertilization. Larvae were then fed 4 cells/μl of the unicellular alga *Rhodomonas lens* or 12 cells/ul *T-iso*. Water in cultures was changed every 2 days by reverse filtration (see Strathmann, '87). At each water–change, fresh food was added.

TH, TH metabolite and thiourea effects on larval development and metamorphosis

In order to estimate T4 effects on development to metamorphosis and metamorphic competence, larvae in cultures were exposed to four concentrations of T4 and the control (no T4) 2 days after fertilization until metamorphosis. Primary T4 stocks [L-Thyroxin (Sigma, St. Louis, MO); T-1776] were prepared at a concentration of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M (Heyland and Hodin, 2004) and then diluted 1:10^4 for the final concentrations of 10^{-10}, 10^{-11}, 10^{-12} and 10^{-13} M (THYROXINE 10^{-10} M; THYROXINE 10^{-11} M; THYROXINE 10^{-12} M; THYROXINE 10^{-13} M) in the culture jars (note that for this experiment we used 1 l glass jars filled with 800 ml MFSW). We monitored development in larvae by removing replicate larvae from culture jars and determining their developmental stage on living specimens. When larvae were considered competent for metamorphosis (for definition of metamorphic competence see Heyland and Hodin, 2004), water in 1 l jars was reduced to 100 ml by reverse filtration (Strathmann, '87) and 4 ml of 1 M KCl was added resulting in 40 mM excess final KCl concentration. Jars were screened for metamorphosed *L. variegatus* after 4 hr. This procedure was repeated 4 times every 2 days. We estimated timing to metamorphic competence in larvae by calculating the cumulative percentage of metamorphosis for the dates listed above. Once the threshold of 20% cumulative percentage metamorphosis was overcome, we consider larvae in a replicate metamorphically competent.

For a broad comparison of the effects of the TH metabolite and TH synthesis inhibitor thiourea in development of morphological structures, we cultured larvae to the developmental stage when the juvenile rudiment was clearly flattened and juvenile spicules were present (see also Chino et al., '94). About 24 hr before exposure, we reared larvae in the complete absence of food to drain the stomachs of any algal diet. We then distributed larvae into individual wells of 12-well plates filled with 4 ml of MFSW and exposed them to one of the following treatments: CONTROL (no chemicals added), THYROXINE [10^{-10} M thyroxine (Sigma:

T-1776)], RESCUE (10^{-10} M thyroxine+10^{-3} M thiourea), THIOUREA (10^{-3} M thiourea), L-TYROSINE (2×10^{-10} M L-tyrosine [Sigma: T9040-9]), NaI (4×10^{-10} M NaI [Sigma: S2179]), L-TYROSINE+NaI (2×10^{-10} M L-tyrosine+4×10^{-10} M NaI), NaCl (4×10^{-10} M NaCl). At this point we took two images from each individual larva that was mounted alive on a microscope slide with sufficient MFSW. Each image was taken at a different magnification ($10\times$, $20\times$) in order to be later able to measure stomach size (SS) and rudiment size (RS), respectively. Note that each treatment was replicated 12 times (i.e., one entire well-plate per treatment). On April 15, we removed each individual larva carefully from the well plate and photographed it in the same way as on April 11. On April 16, we removed larvae from the well plate and flattened larvae underneath cover slides and imaged larval and juvenile skeletal structures. All images were analyzed using imageJ software (http://rsb.info.nih.gov/ij/). We measured postoral arm length (PO), postdorsal arm length (PD), the SS and RS. SS and RS were calculated as the square root of the cross-sectional area of an ellipsoid (using stomach length, stomach width and rudiment length, rudiment width, respectively, as the axes of the ellipsoid).

Iodine incorporation and TH synthesis

We exposed larvae at the developmental stage when adult spicules had formed (see also above) for 8 hr in 12-well plates to experimental treatments. Note that before this exposure we cultured larvae for 24 hr in the complete absence of food to drain the stomachs from any algal food. We placed 50 randomly chosen larvae into each well, containing 4 ml of solution (10^{-3} M thiourea and the control, respectively). All solutions were made up in SW^{125} (MPFSW with I^{125} at 51,937 dpm; Carrier-free specific activity of I^{125} was 642.8 GBq/mg). For each treatment we used 6 wells. After the exposure, larvae from three wells were pooled together in one test tube resulting in two independent replicates per treatment (150 larvae per replicate). Specimens were washed 5 times with fresh MFSW until the radioactivity in the supernatant was below 30 dpm (counted on ssMPD instrument see below). Between each wash larvae were centrifuged at 1,980*g* for 3 min and kept on ice. *A. californica* juveniles were processed in a similar way except that we used 10^{-2} M thiourea in the inhibitor treatment.

To test whether I^{125} that the larvae had incorporated was built into T4, we prepared samples for TLC. We added 1 ml of ice-cold MeOH to each sample after the sample was counted (samples containing I^{125} were counted on a ssMPD instrument [BioTraces, Inc., Herndon, VA] in standard mode. In standard mode, digital signal processing is used to distinguish the I^{125} decay-specific characteristics from those of background events to give a background equivalent to 5 DPM of I^{125} with about 45% efficiency) and let it stand at 4°C overnight. After vortexing all samples at full strength for 2 min, we centrifuged them at 1,980*g* for 10 min and collected the supernatant. Then we spiked the samples with 100 µl non-radioactive 10^{-4} M T4 (thyroxine; Sigma-Aldrich T-1774) and T3 (3,3″,5-triiodo-L-thyronine; Sigma: T2877) and then concentrated in a speed-vac to complete dryness. The dry pellet was redissolved in 30 µl 0.01 N NaOH. Note that usually not all the salt crystals redissolved. All 30 µl (excluding the crystals) were loaded on a TLC plate (Whatman LK5D silica gel 150A with fluorescence marker; Whatman ♯4851-840) and run for 1.5 hr in a 2-methylbutanol/*t*-butyl alcoholy/25%NH3/acetone, 7:14:14:56, vol/vol solvent. We visualized the cold T4 and T3 markers under UV light on a BioRad™ Flour-S MultiImager system and radioactive bands on a Molecular Dynamics™ Phosphorimager SI. Overlaying the UV image with the one from the phosphor imager allowed us to compare the radioactive bands to our THs standards.

TH measurements in sea urchin larvae and Aplysia hemolymph

We collected *Aplysia* hemolymph samples and sea urchin larval samples (40 larvae per sample). In case samples were not immediately processed we kept them at −80°C. Note that samples were never kept longer at −80°C than 2 months. Then we defrosted samples on ice, and added 5 volumes of 100% ice-cold MeOH. Extraction was done at 4°C overnight. We then centrifuged samples at 3,000 rpm for 10 min at 4°C. We decanted the liquid upper phase and kept the pellet for protein analysis. The upper phase was brought to complete dryness in a Speed-Vac™ and then resuspended in 50 µl 0.01 N NaOH. The pellet was redissolved in 100 µl 1 N HCl at 60°C for 1 hr and then vortexed at full strength for 1 min per sample.

For TH analysis we used the ELISA KIT (Total Thyroxine (Total T4) ELISA Kit Alpha Diagnostic International, Inc.; TX, USA) following the

manufacturer's instructions. Note that in addition to the standards provided in the kit we used our own standards made up in 0.01 N NaOH for better comparison. For Protein analysis we used Pierce™ micro-BCA kit using manufacturer's instructions.

Gene cloning and in situ hybridizations

Two pools of double-stranded cDNA from the sea urchin *L. variegatus* larval stages and *A. californica* cerebral ganglia were isolated and amplified following protocols of the Clontech Smart-PCR cDNA synthesis kit (Clontech Laboratories, Mountain View, CA, USA) as described in (Matz, 2003). We ligated cDNA to double-stranded adaptors (Marathon cDNA Amplification Kit, Clontech). For cloning of LvTPO (*L. variegatus* TPO), we selected conserved amino acid sequences among peroxidases to choose the sites for PCR primers. We choose sense [TPO-F: ACIGCIGCITT(TC)(CA)GITT(TC)GGICA, corresponding to the amino acid sequence TAAFRFGH] and the antisense [TPO-R: GGIA(AG)ICC(AG)TG(AG)TCIC(GT)ICCIC(GT)(TC)TG, corresponding to the amino acid sequence QRGRDHGLP] degenerate primers based on (Ogasawara et al., '99). For AcaTPO (*A. californica* TPO), we then used rapid amplification of cDNA ends (RACE) to generate 5′ and 3′ PCR products following procedures previously described in Matz et al. (2003). We cloned Gel purified PCR products into the pT-Adv Vector (Clontech TA cloning) and sequenced it at the Whitney Laboratory, St. Augustine, FL.

We fixed organismal samples in 100 mM HEPES, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA for 24–48 hr and dehydrated them in 50% ethanol, then 80% ethanol (30 min each) and stored at −20°C in 80% ethanol until used for in situ hybridizations. We synthesized digoxigenin labeled (DIG) antisense probes from linearized plasmids according to the protocols supplied with the DIG RNA Labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Whole-mount in situ hybridizations were performed by a similar protocol to that of Swalla et al., ('94). We washed samples with phosphate buffered saline with 0.1% Tween 20 (PBT) then treated with 10 mg/ml Proteinase K in PBT at 37°C for 10 min. We stopped the reaction in 2 mg/ml glycine in PBT, and washed with PBT. Samples were post-fixed in 4% paraformaldehyde in PBS, washed with PBT and treated with 0.25% anhydrous acetic acid in 0.1 M triethanolamine (pH 8.0) prepared just before use. We hybridized samples overnight at 45°C with LvTPO antisense full length probe and used the sense probe as a negative control. We then washed with 2 × SSC at 45°C and treated with 20 mg/ml RNase at 37°C. Samples were blocked in 0.1% blocking reagent in PBT, then incubated in 1/2,000 anti-DIG-AP in PBT, both from the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals). AP detection buffer contained levamisole and NBT/BCIP. After the desired staining was reached, samples were rinsed in PBS. Samples were then mounted in benzyl alcohol:benzyl benzoate after being dehydrated through a series of ethanol washes: 30%, 50%, 80%, 90%, 100% along with two washes in benzyl alcohol:benzyl benzoate 1:1. Note that for all these procedures we cultured larvae for 24 hr in the complete absence of food to drain the stomachs from any algal food (cDNA synthesis and sample fixation). All in situ hybridizations were replicated 3 times independently (i.e., on three different days using three new probes).

Phylogenetic analysis

Amino acid sequences of the peroxidases catalytic domains were aligned using default settings in ClustalX (Thompson et al., '94). No manual adjustment of the alignment was necessary (Appendix A). For a complete list of sequences see Table 1. Phylogenetic analysis was performed using maximum-likelihood, parsimony and Bayesian inference. For the maximum-likelihood analysis we used Tree-Puzzle software (Schmidt et al., 2002). JTT model of amino acid evolution (Jones et al., 1992) was used for maximum-likelihood distance calculation (parameters estimated from the dataset), gamma distribution of sites variability was assumed (alpha parameter estimated from the dataset) and 10,000 puzzling steps were done to obtain support values. For the parsimony analysis, we used PAUP* 4.0b10 for Windows (Sinauer Associates, Inc., Sunderland, MA, USA). All characters were equally weighted and unordered. Gaps were treated as missing data. The analysis was performed using 10,000 random addition sequence replicates, holding 10 trees at each step and TBR branch swapping algorithm. Clade support was estimated with 1,000 bootstrap replicates (100 replicates of random addition with 10 trees held at each step). Bayesian inference was carried out using MrBayes, ver. 3.0B4 (Huelsenbeck and Ronquist, 2001). We used

TABLE 1. Genes used for phylogenetic analyses

Abbreviation	Protein name	Organism	Database ID	Length	Domain
PERT:HALOR	Thyroid peroxidase	*Halocynthia roretzi* (Ascidian)	BAA76689 (gb)	918	214–716
PERT:HUMAN	Thyroid peroxidase	Human	P07202 (sp)	933	201–711
PERT:MOUSE	Thyroid peroxidase	Mouse	P35419 (sp)	914	195–699
PERT:CIONA	Thyroid peroxidase	*Ciona intestinalis* (Ascidian)	BAA76688 (gb)	909	200–703
PERT:RAT	Thyroid peroxidase	*Rat*	P14650 (sp)	914	195–699
PERT:PIG	Thyroid Peroxidase	*Pig*	P09933 (sp)	926	201–709
PERS:MOSQ1	Salivary peroxidase	*Anopheles albimanus* (Mosquito)	AAD22196 (gb)	591	75–568
RIBON:XENLA	Polysomal ribonuclease 1	*Xenopus laevis* (Frog)	AAC94959 (gb)	713	195–689
PERNT:PACL	Peroxinectin	*Pacifastacus leniusculus* (Signal crayfish)	JC4397 (pir)	818	293–787
PERDSN:HUMAN	Peroxidasin homolog	*Human*	BAA13219 (gb)	1,496	808–1,308
PERDSN:DROME	Peroxidasin homolog	*D. melanogaster* (Fruit fly)	S46224 (gb)	1,535	831–1,329
PERDSN1:CELE	Peroxidasin homolog	*C. elegans*	CAA91994 (gb)	1,328	719–1,225
PERH2N:CELE	Peroxidase homolog 3N	*C. elegans*	CAA88963 (gb)	1,490	205–676
AcaTPO	Peroxidase homolog	*Aplysia californica* (Sea hare)	AAT90333 (gb)	560	1–560
PERH:MOSQ1	Peroxidase homolog	*Aedes aegypti* (Yellow fever fly)	AAC97504 (gb)	683	138–642
LvTPO	Peroxidase homolog	*Lytechinus variegatus* (Sea urchin)	AAT90332 (gb)	678	49–548
PERH:BRANB	Peroxidase homolog	*Branchiostoma belcheri* (Lancelet)	BAA83376 (gb)	764	231–738
PERH:DROME	Peroxidase homolog	*D. melanogaster* (Fruit fly)	Q01603 (sp)	690	150–652
PERH:PSEDA	Peroxidase homolog	*Pseudomonas alcaligenes* (Bacterium)	AAC83355 (gb)	913	100–614
PIOX:TABAC	Pathogen induced oxygenase	*Nicotiana tabacum* (Tabaco)	CAA07589 (gb)	643	130–623
PERO:HEMP	Ovoperoxidase	*Hemicentrotus pulcherrimus* (Sea urchin)	BAA19738 (gb)	814	194–686
PERM:HUMAN	Myeloperoxidase	Human	P05164 (sp)	745	223–720
PERM:MOUSE	Myeloperoxidase	Mouse	P11247 (sp)	718	197–694
PERI:SEPO	Melanogenic peroxidase	*Sepia officinalis* (Cuttlefish)	CAA72331 (gb)	926	369–852
PERI:EUPS	Light organ peroxidase	*Euprymna scolopes* (Squid)	AAA16244 (gb)	894	337–820
PERL:BOVIN	Lactoperoxidase	Bovine	P80025 (sp)	712	188–686
PERL:HUMAN	Lactoperoxidase	Human	P22079 (sp)	712	188–686
PERE:MOUSE	Eosinophil peroxidase	Mouse	CAI25724 (gb)	716	196–692
PERE:HUMAN	Eosinophil peroxidase	Human	P11678 (sp)	715	195–691
PGH2:HUMAN	Cyclooxygenase-2	Human	P35354 (sp)	604	156–568
PGH1:HUMAN	Cyclooxygenase-1	Human	P23219 (sp)	599	169–581

The first column corresponds to the name used in the phylogenetic tree and the alignment (Fig. 4B; Appendices A and B). The second column is a characterization of the peroxidase. Column three describes the organism from which the gene originates. Column four is the database accession number (sp: SwissProt; gb: Genbank; pir: Protein Information Resource). Column five indicates the number of amino acids of the gene and column 6 indicates the region in which the catalytic domain was identified and used for the phylogenetic analysis.

a fixed JTT model for amino acid evolution (Jones et al., 1992). Each Markov chain was started from a random tree and run for 2×10^6 generations, sampling every 100th generation from the chain. Each run comprised four differently heated chains. The analysis resulted in the accumulation of 60,000 trees. The first 30,000 generations of data (i.e., 3,000 trees) were discarded (''burned'') for the posterior probabilities calculation. Three independent runs were performed. For parsimony and maximum-likelihood analyses we used the following four taxa as outgroups: PERH:PSEDA, PIOX:TABAC, PGH2:HUMAN and PGH1:HUMAN. For Bayesian analysis we used PIOX:TABAC (Pathogen induced oxygenase from *Nicotiana tabacum* Genbank accession number (CAA07589)).

Data analysis

Data were organized and analyzed in Excel^{TM} and SPSS^{TM}. Statistical comparisons between the experimental treatments and the controls were done using student's *t*-test, ANOVA with simple contrast or MANOVA. For all analyses we used SPSS^{TM}. Results are presented as: mean difference (treatment value minus control value) $\pm$ 1SE; *P*-value. If the mean difference is positive this means that the value in the experimental treatment was larger than the value in the control. All *P*-values are from null hypotheses testing that the mean difference mentioned above equals 0. Information on *A. californica* ESTs was derived from the *Aplysia* neuronal transcriptome (Moroz et al., unpublished data). EST and genomic information

from the sea urchin *Strongylocentrotus purpuratus* was derived from NCBI.

RESULTS

Iodine incorporation and TH synthesis in two invertebrate species is inhibited by thiourea

We exposed larval sea urchins and juvenile *Aplysia* to I^{125} in order to test whether (1) iodine is incorporated into these organisms and (2) incorporated iodine is used for TH synthesis (T4 and triiodotyrosine). Sea urchin larvae incorporated I^{125} (Fig. 2A and B). Thiourea, a TPO inhibitor, blocked iodine uptake in a dose-responsive manner in the sea urchin (Fig. 2A). However, $KClO_4$, known as a competitive inhibitor of the sodium iodine symporter (Dai et al., '96; Eskandari et al., '97), did not have any effects on iodine incorporation in sea urchins (Fig. 2B).

Our results from the TLCs confirm that both the sea urchin larvae and *Aplysia* juveniles used incorporated I^{125} to make THs (Fig. 2C and D). Interestingly, *Lytechinus* larvae predominately synthesize T4 (Fig. 2C) while *Aplysia* juveniles primarily synthesize T3 (Fig. 2D). Thiourea inhibited TH synthesis in both *Aplysia* and sea urchins (Fig. 2C and D).

Finally, we were able to detect T4 and T3 in sea urchin larvae and hemolymph of adult *Aplysia* (Fig. 2E) using ELISA. Note that all measurements are standardized to the protein content of the samples.

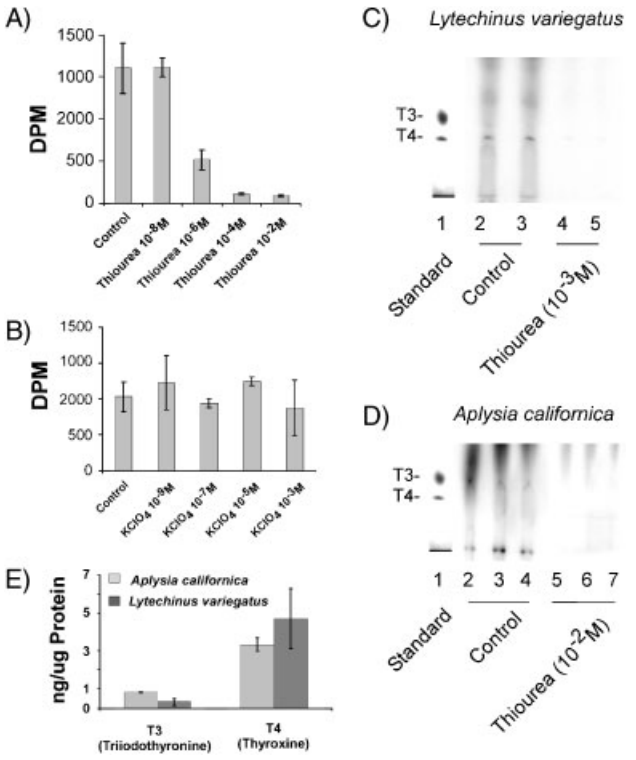

Fig. 2. *Lytechinus variegatus* larvae and *Aplysia californica* juveniles incorporate I^{125} (radioactively labeled iodine) and synthesize iodinated tyrosines (i.e., T4 and T3). (**A**) Thiourea inhibits iodine (I^{125}) uptake in *Lytechinus* variegates; (**B**) $KClO_4$ (another thyroid hormone synthesis inhibitor, inhibiting the Na/I symporter in vertebrates, for details see text) has no effect on iodine uptake. (**C**) Incorporated iodine is used to synthesize thyroxine (T4) in *Lytechinus variegatus* (Control; lanes 2 and 3, standard in lane 1); (**D**) Incorporated iodine is used to synthesize T3 in *Aplysia californica* (Control, lanes 2–4, standard in lane 1). (**E**) We measured T4 and T3 in sea urchin (*Lytechinus*) larvae and *Aplysia* juveniles using ELISA and found both hormones present in both species in comparable concentrations. Note that all measurements are standardized to the protein content of the samples. T4 was present at higher concentrations in both species. DPM: decays per minute. In both *Aplysia* and *Lytechinus* neither T4 nor T3 was detected when larvae and juveniles were exposed to thiourea before extraction.

THs accelerate development to metamorphosis in sea urchin larvae while thiourea acts in an inhibitory manner

First, we tested T4 effects on pre-metamorphic stages and attainment of metamorphic competence of the sea urchin *L. variegatus* (Fig. 3A and B). Secondly, we tested whether iodine, tyrosine, thiourea (a TPO inhibitor) and T4 result in morphological changes in sea urchin larvae (Fig. 3C, D). T4 significantly accelerated occurrence of juvenile spines (Fig. 3A) and attainment of metamorphic competence (Fig. 3B) in larvae that were continuously exposed to the hormone during development. The number of adult skeletons was significantly higher in the THYROXINE 10^{-10} M (6.46 ± 1.91; $P = 0.001$) AND THYROXINE 10^{-11} M (4.05 ± 1.91; $P = 0.038$) treatment 9 days after fertilization compared to the control using ANOVA with simple contrast (Fig. 3A). Metamorphic competence was reached significantly earlier (approximately 15%) in the THYROXINE 10^{-10} M treatment compared to the control (-3.50 ± 1.45 days; $P = 0.03$) (Fig. 3B). Note that we used a threshold value of 20% competence for this analysis (data not shown).

We then tested the effects of various THs and TH metabolites on larval and juvenile morphology using well-plate experiments (for experimental design see materials and methods). T4 lead to a significantly stronger relative reduction of SS (-0.21 ± 0.09; $P = 0.02$), and absolute reduction

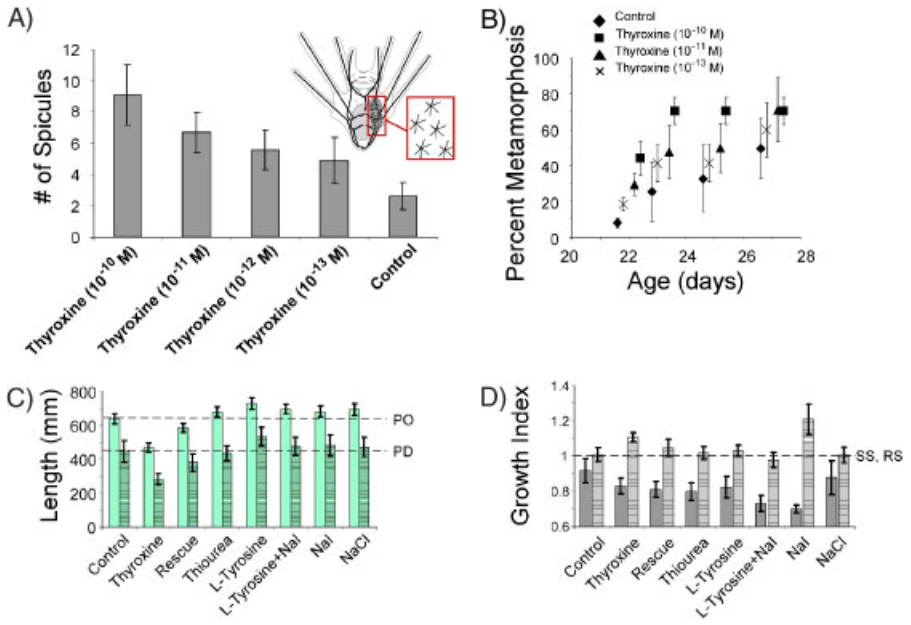

Fig. 3. Thyroxine (T4) accelerates larval development (A) and development to metamorphosis (B). (**A**) Larvae of *Lytechinus variegatus* were treated with different concentrations of T4 during larval development ranging from 10^{-10} to 10^{-13} M. We observed a significant increase in number of spicules that formed in the adult rudiment. We showed earlier that this spicule formation is a good indicator for juvenile development in pluteus larvae (see Heyland and Hodin, 2004). (**B**) T4 also significantly accelerated development to metamorphosis (analysis see text) measured in the time it takes larvae to reach metamorphic competence threshold of 20% (please note that measurements were done at the same time points 22, 23, 25, 27 days after fertilization). (**C**,**D**) We exposed larvae to seven different treatments and the control in order to test whether iodine and/or tyrosine simulate the effect of T4 on larval characters (C), the stomach (D) and the juvenile rudiment (D) and whether thiourea has an inhibitory effect on these structures as it was found in other echinoid larvae. Thiourea did not have any effects on larval and juvenile structures while T4 did. The rescue treatment (T4+thiourea) however was not significantly different from the control either. We conclude from these findings that thiourea has an opposite effect on morphological characters than T4. We also found that NaI significantly decreases stomach size and so does T4 (for details on statistical analysis see text). No shading in (C): postoral arm length; horizontal shading in (C): posterodorsal arm length; no shading in (D): stomach size; horizontal shading in (D): rudiment size. Growth index indicates SS or RS after experimental exposure divided by SS or RS before exposure, respectively. Dashed lines indicate the o-change isoclines. For PO and PD arm length this corresponds to the length of the arms in the CONTROL while it corresponds to 1 for stomach size and rudiment size (indicating no change).

of PO arm length (-168.98 ± 43.17; $P < 0.001$) and PD arm length (-169.90 ± 74.20; $P = 0.02$) and a significant relative increase in juvenile size (0.21 ± 0.07; $P = 0.005$) all relative to the control. We did not find however any significant difference between the THIOUREA treatment (10^{-3} M thiourea) and the CONTROL and the RESCUE treatment (RESCUE [10^{-10} M T4+10^{-3} M thiourea]) and the CONTROL indicating an inhibition of T4 effects by thiourea in terms of larval morphology. Furthermore, we did find a significant relative decrease of SS (-0.19 ± 0.09; $P = 0.04$) in the NaI treatment. In summary, we found that T4 treatment results in decreased growth and development of larval structures and an increase in juvenile structures. These effects of T4 are reversed by the addition of the thyroid hormone synthesis inhibitor thiourea to our cultures.

Cloning of peroxidases from echinoderms and Molluscs and phylogenetic analysis

As a first step to elucidate the mechanisms of TH synthesis in non-chordate animals, we searched for TPO orthologous genes using degenerate primers and database screens. We cloned two partial sequences of peroxidases closely related to peroxidasin and TPO from *A. californica* (AcaTPO: AY605096) and the sea urchin *L. variegatus* (LvTPO: AY605095; Fig. 1C). Both sequences were submitted to Genbank.

Figure 4A is a schematic representation of the domain structure derived from the complete alignment of *Drosophila melanogaster* peroxidasin and several TPOs. The alignment with details on conserved residues can be found in Appendix A. The sequenced portion of LvTPO encodes 703

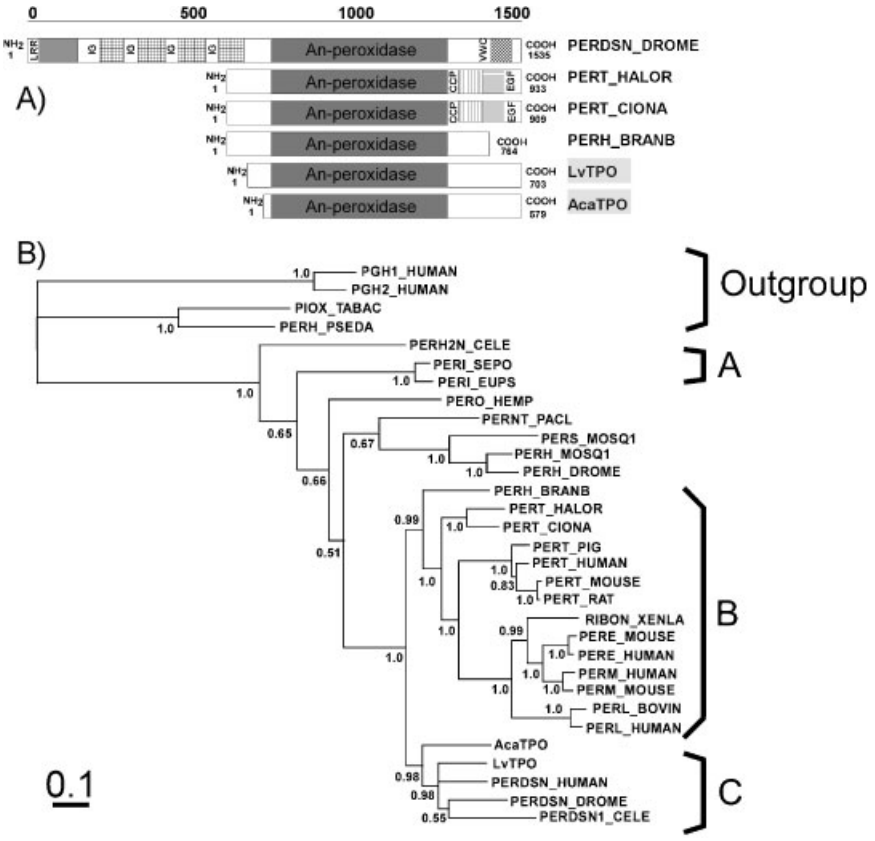

Fig. 4. **(A)** Schematic result of sequence alignment with putative domain structure. Partial sequences of AcaTPO and LvTPO and their alignment with other peroxidases are represented in Appendix 1. Schadings in the schematic alignment indicate conserved motifs and are from 5′ to 3′: LRR: Leucine-rich repeats; IG: Immunoglobulin domain cell adhesion molecule (cam) subfamily; An-peroxidase: animal haem domain; CCP: complement control proteins; EGF: Calcium-binding EGF-like domain; VWC: von Willebrand factor type C domain **B**) Bayesian analysis using amino acid sequence of catalytic domains confirms AcaTPO and LvTPO as peroxidases closely related to peroxidasins (Clade C). Clade C represents a sister clade to thyroid- myelo-, lacto-, eosinophil-peroxidases (Clade B). All peroxidases used for the alignment are summarized in Table 1. Note that parsimony and maximum likelyhood analyses (see Appendix 2) resulted in similar tree topology as the bayesian analysis shown here. Values underneath branches represent posterior probabilities.

amino acid residues. The catalytic domain extends from position 74 to 574 and includes all residues necessary for peroxidase function and critical for thyroid hormone synthesis. These are proximal and distal histidine (110, 356), arginine (259) and asparagine (440). The Ca^{2+}-binding domain appears to be only partially conserved in *L. variegatus* [residues T(189), Y(191), D(193), S(195)]. While three residues are completely conserved [T(189), D(193) and S(195)] position 191 is Y in the sea urchin, instead of F as in other TPO genes described. AcaTPO is 576 amino acids long and has also all major domains necessary for peroxidase function conserved. These are proximal and distal histidine in positions 25 and 273, Ca^{2+} binding domain in positions 104 (T), 106 (F), 108 (D) and 110 (S). Finally arginine is found in position 77 and asparagines in position 357. In the sequenced portion of LvTPO and AcaTPO, we were not able to detect any of the complement control protein (CCP) modules (also known as short consensus repeats (SCRs) or SUSHI repeats) towards the 3′ end of the gene as they can be found in human (PERT:HUMAN) and *C. intestinalis* (PERT:CIONA) TPOs. Furthermore, we were not able to find a second calcium-binding EGF-like domain in the partial sequence of LvTPO and AcaTPO.

In order to evaluate the phylogenetic position of AcaTPO and LvTPO among other peroxidases, we performed three different analyses: parsimony, maximum-likelihood and Bayesian. Amino acid sequences used in the phylogenetic analysis are summarized in Table 1. The tree resulting from the Bayesian analysis is presented in Figure 4B. The alignment of the catalytic domain is provided in Appendix A, and the same alignment in nexus

format is provided in Appendix C. For parsimony analysis all characters were weighted equally and gaps were treated as missing values. From a total of 599 characters, 108 were parsimony uninformative and were excluded. This analysis resulted in two maximum parsimony trees (L = 4,732; CI = 0.65; RI = 0.61). All trees showed very similar results. Bayesian and tree puzzle analysis found significant support for Clades A–C, while parsimony did not identify peroxidasins, LvTPO and AcaTPO as a monophyletic group. Clade A consists of melanogenic peroxidase from the cuttlefish *Sepia officinalis* and light organ peroxidase from the squid *Euprymna scolopes*. Clade B consists of lacto-, myelo-, eosinophil and TPOs. The position of polysomal ribonuclease 1 from the frog *Xenopus laevis* and a peroxidase ortholog from the lancelet *B. belcheri* are consistent between maximum-likelihood and Bayesian but not compared to parsimony. Clade C consists of LvTPO and AcaTPO together with peroxidasins from *Drosophila*, *C. elegans* and humans. Trees resulting from the maximum likelihood and parsimony analyses are presented in Appendix B.

Thus, AcaTPO and LvTPO can be characterized as peroxidases based on their conserved residues. Based on their sequence similarities and topology of the phylogenetic tree, we suggest that both AcaTPO and LvTPO can be classified as peroxidasin orthologs.

Temporal and spatial transcription pattern of TPO orthologs in Aplysia and sea urchin

We used probes from partial sequences to characterize expression patterns of LvTPO in sea urchin larvae (Fig. 5A–F) and of AcaTPO in the CNS of *Aplysia* (Fig. 5G–I).

Figure 5A and D presents a planktonic larva of *L. variegatus* 7 days after fertilization and Figure 5B and E is a pre-metamorphic stage of *L. variegatus* 11 days after fertilization. In both stages we detected expression of LvTPO in distinct cell clusters around the mouth region (M), in the stomach (St) and the ectoderm of the juvenile rudiment (R). At later developmental stages (Fig. 5B) we also detected transcription in the pedicellariae (Pe), characteristic skeletal structures that start forming during late larval development and are maintained in the adult sea urchin. No staining was detected in control larvae where we used LvTPO sense probe (Fig. 5C). Note however that some control larvae showed background staining in the arms and very weak staining in the stomach region (not shown). Figure 5F summarizes the transcription patterns resulting from the antisense probe schematically.

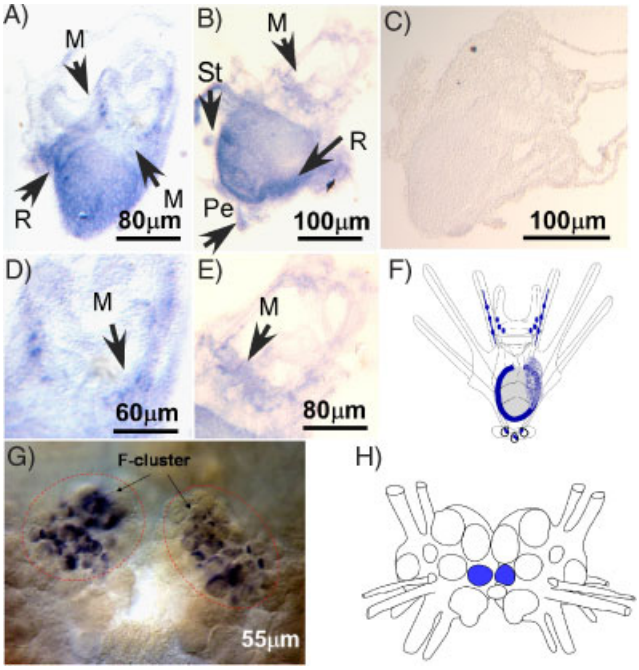

Fig. 5. We performed in situ hybridizations on *Lytechinus variegatus* larval stages (**A**–**F**) and the central nervous system of *Aplysia californica* (**G** and **H**) using LvTPO and AcaTPO RNA probes, respectively. We detected ubiquitous expression of LvTPO throughout development in the stomach region and the mouth region, the ectoderm of the juvenile rudiment and the stomach. Some of the more specific and pronounced expression occurs in specific cell clusters around the mouth as shown in the close-up images presented in (D,E). (C) Control (sense probe) larvae presented in (C) do not show any staining. Note however that some control larvae showed background staining in the arms and very weak staining in the stomach region (not shown); larva in (A,D) is 7 days old, larva in (B,E) is 11 days old. (F) summarizes schematically the transcription patterns found in (A,B). (G) Neurons of two symmetrical F-clusters (a proposed neuroendocrine center located in the cerebral ganglia of *Aplysia*) stain positive in in situ hybridization with antisense probe (localization of cluster within the cerebral ganglion is schematically represented in (H). (**J**) AcaTPO sense probe did not produce any positive staining (data not shown). R: juvenile rudiment; M: mouth; PE: Pedicelaria; St: stomach. The arrows in the figure showing larvae of *Lytechinus variegatus* and the cerebral ganglia of *Aplysia californica* are pointing anterior. The cerebral ganglia of *Aplysia californica* are shown from the dorsal side.

In the *Aplysia* CNS, expression of the AcaTPO was detected in the cerebral ganglia, specifically in the area of neurosecretory F-cluster (Fig. 5G). No staining was detected in the control (not shown). The overall morphology of the cerebral ganglion and the F-cluster are depicted in Figure 5H.

Database analysis

We screened EST and genomic databases of *S. purpuratus* (NCBI) for TH signaling-related transcripts. We cloned three new transcripts from the sea urchin potentially involved in TH signaling. These are sulfotransferase (Sp_SULT; DQ176319), retinoic X receptor (Sp_RXR; DQ176321 same sequence as XM_779153) and a new isoform of chicken ovalbumin upstream promoter (Sp_COUP01; DQ176320). Finally, several components of the TH signaling pathway have now been annotated from the newly released *S. purpuratus* genome. These are: thyroid hormone receptor (TR) (gb XP_79257), TR-associated and interacting proteins (XP_795219, XP_780945, XP_786427, XP_796020, XP_781449, XP_791710, XP_785120, XP_791710, XP_790753, XP_795235, XP_790569, XP_790999, XP_792847, XP_789723, XP_784390, XP_780593, XP_787043, XP_780442, XP_780362, XP_783595), thyrotropin receptor precursor (XP_782262).

DISCUSSION

Data presented here provide evidence for thyroid hormone-related signaling in the Mollusc *A. californica* (sea hare) and the echinoderm *L. variegatus* (sea urchin). Our analysis shows that THs affect the development to metamorphosis in sea urchins significantly and that both the sea hare and the sea urchin can synthesize THs endogenously. Furthermore, we cloned two new peroxidases from these two species and the expression patterns in larvae and adults suggest a possible role of these genes in development and metabolism. Together, this new evidence provides support for the hypothesis that endogenous TH synthesis is not a synapomorphy of chordates. Finally we propose a new model of endogenous TH synthesis via peroxidases among non-chordate metazoa.

Endogenous TH synthesis in the sea hare and sea urchin

Numerous reports have been published on TH-related function in various invertebrate phyla (Fig. 1, *reviewed in* Eales, '97). However, the mechanisms and specific TH signaling pathways are presently unknown. Recent studies have documented TH-like signaling among Echinodermata (Chino et al., '94; Johnson and Cartwright, '96; Suyemitsu et al., '97; Johnson, '98; Saito et al., '98; Suyemitsu, 2000; Hodin et al., 2001; Heyland and Hodin, 2004) a sister taxon to chordates. These data suggest that TH-like compounds play a critical role in echinoderm larval development and metamorphosis including preliminary evidence for endogenous hormone synthesis (Chino et al., '94; Saito et al., '98; Heyland and Hodin, 2004), involvement of TH signaling in the phenotypic plastic response to food of echinoid larvae (Heyland and Hodin, 2004) and the timing of attaining metamorphic competence of echinoderm larvae (Chino et al., '94; Suyemitsu et al., '97; Saito et al., '98; Suyemitsu, 2000; Hodin et al., 2001; Heyland and Hodin, 2004).

Our TLC analysis reveals that the sea urchin and the sea hare incorporate iodine into TH similar to T4 and T3, respectively, which we interpret as evidence for endogenous TH synthesis. These findings are further backed up by ELISA measurements of THs in larvae and adult of these organisms. Intriguingly the synthesis of THs is inhibited by the TPO inhibitor thiourea. It is not clear though on what level the synthesis of these molecules is inhibited. Since thiourea treatment not only decreases the amount of hormone synthesized but also decreases the intensity of the non-specifically incorporated iodine, it is possible that other processes involved in iodine uptake and/or binding are affected by the inhibitor. The fact that $KClO_4$ does not affect the uptake of iodine tells us that iodine uptake/concentration mechanisms in echinoderms are different from those found in vertebrates. $KClO_4$ is a potent competitive inhibitor of iodine uptake into the thyroid gland that is mediated via the sodium-iodine symporter. In fact it is conceivable that in marine invertebrate larvae, iodine uptake from seawater occurs via epithelial diffusion as it is well known from the gut epithelium of humans and other vertebrates.

Thiourea effects on sea urchin larvae from this study were very different from effects that we previously described on larvae of the sand dollar *D. excentricus* (Heyland and Hodin, 2004). While thiourea had a significant effect on arm length, SS and RS in *D. excentricus* larvae (Heyland and Hodin, 2004), these effects were either absent and/or much weaker in the sea urchin. We interpret this as evidence that endogenous sources of hormones are more important for sand dollar larval development than for sea urchin larval development. These findings are particularly interesting in the context of our previously stated hypothesis that feeding larvae from different echinoderm clades have different abilities to

synthesize THs endogenously and that feeding larvae with an increased capacity for endogenous TH synthesis can be found specifically in clades in which non-feeding development evolved frequently (Heyland and Hodin, 2004; Heyland et al., 2005). However, alternatively this difference could also be explained by the difference in experimental design between the two studies. While we exposed sand dollar larvae continuously to thiourea in the presence of algal food, sea urchin larvae in the present study were exposed to the inhibitor in the absence of food over a limited period of time. The advantage of the design used in this study is that indirect effects of inhibitor on algae can be excluded and in fact it could be such effects that are ultimately responsible for the differences seen between sand dollar and sea urchin larvae. We propose to use the design used in this study for future experiments to exclude the indirect effects described above.

Iodine simulates the effects of T4 on rudiment and stomach morphogenesis in the sea urchin. Both iodine and THs can originate from exogenous sources. Chino et al. ('94) first emphasized the presence of THs in unicellular algae that are commonly used as larval nutrition. Our recent study confirmed measurements of THs in algae (Heyland and Moroz, 2005). Furthermore, the ability of endogenous hormone synthesis, although present, appears to be reduced in feeding larvae, suggesting that some of the hormone may be supplied from exogenous sources such as algae. Iodine concentration is generally much higher in marine than in terrestrial environments, but even in the oceans, iodine concentrations can vary significantly (Truesdale and Bailey, 2002). The potentially dual source of hormone and/or iodine on development is reflected in the expression patterns of LvTPO in the stomach and the adjacent surface of the juvenile rudiment. These are also candidate structures through which iodine and THs could be incorporated into the larva.

THs as developmental and metabolic regulators

The effects of T4 on *L. variegatus* are well in line with these previous findings in that we were able to confirm an acceleration of development in the sea urchin that is very similar to the sand dollar *D. excentricus*. This acceleration is not only represented in an earlier attainment of metamorphic competence but also in the earlier appearance of spine precursors in the juvenile rudiment.

Pharmacological effects of THs on *A. californica* have been previously shown: A sodium-phosphate and sodium-sulfate symporter in the *Aplysia* foregut epithelium was stimulated by triiodothyronine (T3) (Gerencser et al., 2002a,b) suggesting that TH may play a role in phosphate and sulfate homeostasis. Our data indicate that in contrast to *L. variegatus* larvae, *Aplysia* juveniles primarily synthesize T3 and not T4. This result suggests that TH signaling in *A. californica* is likely not mediated via TRs. TRs have a very high affinity to T3 and in vertebrates the pre-hormone T4 is converted to T3 only in the target cell which is a crucial mechanism to ensure the specificity of the hormone. In addition to their genomic action, THs can act via a variety of non-genomic pathways that include, for example, MAPK transduction pathways (Davis and Davis, '96). A number of such non-genomic actions are equally responsive to T4 and T3 (Davis and Davis, '96; Hulbert, 2000). Finally, some preliminary recent evidence suggests the presence of a TR ortholog in the sea urchin genome (Genbank accession number XM_784395). Our search of the *Aplysia* neuronal transcriptome (Moroz et al., unpublished data) did not reveal a TR ortholog.

TH signaling in the nervous system

The transcription of AcaTPO in the cerebral ganglion of *A. californica* implies the exciting possibility that THs signal on the level of the CNS in non-chordate deuterostomes. Although we did not establish a functional link between the peroxidase we cloned and TH function it is worth mentioning that the areas of F- and C-clusters of the *Aplysia* CNS, where AcaTPO is expressed, are part of the neurosecretory center that is able to synthesize insulin-like peptides, cerebrin and possibly other peptide hormones yet to be identified (Rubakhin et al., '99). If AcaTPO is in fact involved in TH synthesis, these hormones could be critical players in this neuroendocrine network that integrates information from the environment with internal signals in order to fine tune feeding behavior and metabolic functions. Intriguingly, some expression domains of LvTPO are linked to the larval nervous system. Specific cell clusters in the mouth region and later in development in the pedicellariae (defensive structures of larvae and adults) are both elements of the larval nervous system (Burke, '80, '83) which also express nitric

oxide synthase (Bishop and Brandhorst, 2001). Finally the function of TH-related transcripts on the level of the nervous system has been reported in polychaetes (Marcheggiano et al., '85; Davoli et al., '91). *E. foeta* incorporates radioactive iodine, traces of which were detected in the CNS of the worm specifically between neursecretory cells and the neuropile fibers (Davoli et al., '91). Marcheggiano ('85) also detected thyroglobulin-like immunoreactivity in the nervous system of *Eisenia* which had a distinct expression pattern from cholinesterase a protein that has high sequence similarity to one thyroglobulin subunit.

The detection of THs in hemolymph of *Aplysia* finally indicates that these hormones may also act as circulatory hormones with non-neuronal functions related to metabolism and homeostasis. It is therefore crucial to further elucidate the role of THs in *Aplysia* metamorphosis using an experimental approach as we have successfully applied it to various echinoid larvae (Heyland and Hodin, 2004; Heyland et al., 2004).

Have peroxidases been co-opted for thyroid hormone synthesis?

AcaTPO and LvTPO fall into one clade together with peroxidasin from human, *Drosophila* and *C. elegans*. Our phylogenetic analysis shows that this peroxidasin clade is a sister clade to animal peroxidases (*sensu* Taurog, 1999) that have been only identified in chordates and includes genes coding for thyroid, myelo and lactoperoxidases. *Drosophila* peroxidasin shows many important similarities to vertebrate peroxidases as has been shown by Nelson et al. ('94). In fact, this peroxidase has been shown to synthesize THs in vitro (Nelson et al., '94). Furthermore, we were able to identify proximal and distal histidines in LvTPO and AcaTPO. In vertebrate TPOs, these residues are located on opposite sides of the heme and participate in the heterolytic cleavage of the O–O bond, an essential step for the formation of compound I (Poulos and Fenna, '94). This step is necessary for the oxidation of iodide, critical in thyroid hormone synthesis. In fact, the lack of the proximal histidine in an alternatively spliced form of TPO (TPO-2) has been hypothesized to be the cause why this enzyme cannot synthesize T4 (Niccoli et al., '97). Finally, another site critical for TH synthesis, the Ca^{2+} binding domain, is also well conserved in AcaTPO and LvTPO.

The synthesis of THs via TPOs is generally viewed as a synapomorphy of chordates. Two new TPO orthologs that were recently cloned from two ascidian species (urochordata) provide additional support for this hypothesis (Ogasawara et al., '99). Our new findings suggest however that TH synthesis is not restricted to the chordates. Our search for TPO-related genes in these two clades using degenerate PCR and database searches (*A. californica* neuronal transcriptome, Moroz et al., unpublished data) and genomic databases (*S. purpuratus* genome Genbank) however did not reveal any TPO orthologous genes in these clades. We argued recently that various peroxidases could have been co-opted independently in different metazoan lineages for thyroid hormone synthesis (Heyland and Moroz, 2005) due to the presence of specific conserved residues in the catalytic domain of these genes which are necessary for tyrosine cross-linking and iodination (Taurog and Howells, '66; Nelson et al., '94). Moreover, the oxidation of iodide to iodine is a critical step for scavenging reactive oxygen species that could do potential damage to the cell (Venturi et al., 2000; Heyland and Moroz, 2005). The reaction of iodine with tyrosine residues removes potentially poisonous iodine from the cell. It has therefore been proposed that the production of iodine and the subsequent iodination of tyrosines via peroxidase action represent a mechanism that primarily evolved as a cellular response to deal with iodine from the environment (Venturi et al., 2000; Heyland and Moroz, 2005). It will be therefore interesting for future studies to investigate the hypothesis that various invertebrate peroxidases are involved in TH synthesis and/or metabolism.

EST and genomic screening for TH-related transcripts in Molluscs and echinoderms

Apart from vertebrates, the first molecular insight in understanding TH signaling in basal chordates has been obtained from the sequenced genome of the urochordate *C. intestinalis*. A whole battery of molecules was found that is potentially involved in TH function (Dehal et al., 2002). Moreover *C. intestinalis* possesses an endostyle, the hypothesized homolog to the vertebrate thyroid gland. Ogasawara et al. ('99) have previously provided evidence for the presence of a TPO orthologous gene that is expressed in a specific zone of the ascidian endostyle and could be involved in TH synthesis. We were able to identify and clone several transcripts potentially involved in TH function from the sea urchin

(*S. purpuratus*) genome. The newly released *S. purpuratus* genome furthermore reveals several TH-signaling-related transcripts including TR orthologous genes. Although further analysis is required to confirm the molecular identity of these transcripts, we will here outline some implications of the presence of such elements in Molluscs and echinoderms.

THs signal via TR, factors that regulate transcription upon binding of ligand (TH). These transcription factors recruit various co-regulators for proper regulation of transcription. TRs heterodimerize with retinoic X receptor which we were also able to identify from *S. purpuratus* (DQ176321, see also XP_792571). One putative repressor of TR is chicken ovalbumin upstream promoter (Zhang and Dufau, 2004) that has been previously identified from sea urchins (Chan et al., '92; Kontrogianni-Konstantopoulos et al., '96). We identified a new form of this orphan receptor from *S. purpuratus* (DQ176320). The *S. purpuratus* genome also reveals several TR interacting proteins which might act as co-activators or repressors of TH signaling in echinoderms. In addition to the genomic mode of action of THs via TRs, non-genomic modes of TH action have also been described (Davis and Davis, '96; Brent, '99; Hulbert, 2000) in which T4, T2 or rT3 can have significantly stronger effects than T3. These signaling pathways are usually characterized by the absence of de novo protein synthesis and a much faster mode of action, properties particularly critical for signaling in the nervous system and circulatory system (*for a recent review see* Hulbert, 2000). These non-genomic modes of TH action should be further investigated in non-vertebrate animals.

Deiodinases transform T4 to T3 which in vertebrates then binds to TRs with high affinity (see above). Another pathway that controls the availability of active T3 involves sulfotransferases (SULTs). These enzymes can transform T4 to rT3 but also have high affinity to various other THs (for review see Visser, '94). We did not identify any deiodinases in *Aplysia* and the sea urchin. However, we were able to retrieve the full-length sequence of a SULT from *S. purpuratus* (DQ176319) that shows high sequence similarity to phenol preferring SULTs belonging to subfamily 1A. Enzymes from this family have high affinity to THs (Strott, 2002). Such SULTs could be involved in the regulation of active THs in echinoid and other marine invertebrate larvae by regulating the iodination state of di-tyrosines.

These preliminary data on TH-related transcripts in *Aplysia* and the sea urchin provide a first step towards identification of TH-related genes in marine invertebrates. In situ hybridizations and RNAi will help to understand whether and how these genes are involved in TH-related function in these species.

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