

## SYMPOSIUM

# Active Metabolism of Thyroid Hormone During Metamorphosis of Amphioxus

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**Synopsis** Thyroid hormones (THs), and more precisely the 3,3',5-triiodo-L-thyronine ( $T_3$ ) acetic derivative 3,3',5-triiodothyroacetic acid (TRIAC), have been shown to activate metamorphosis in amphioxus. However, it remains unknown whether TRIAC is endogenously synthesized in amphioxus and more generally whether an active TH metabolism is regulating metamorphosis. Here we show that amphioxus naturally produces TRIAC from its precursors  $T_3$  and L-thyroxine ( $T_4$ ), supporting its possible role as the active TH in amphioxus larvae. In addition, we show that blocking TH production inhibits metamorphosis and that this effect is compensated by exogenous  $T_3$ , suggesting that a peak of TH production is important for advancement of proper metamorphosis. Moreover, several amphioxus genes encoding proteins previously proposed to be involved in the TH signaling pathway display expression profiles correlated with metamorphosis. In particular, thyroid hormone receptor (TR) and deiodinases gene expressions are either up- or down-regulated during metamorphosis and by TH treatments. Overall, these results suggest that an active TH metabolism controls metamorphosis in amphioxus, and that endogenous TH production and metabolism as well as TH-regulated metamorphosis are ancestral in the chordate lineage.

## Introduction

Hormones have wide effects on animal physiological and developmental processes, from reproduction to metamorphosis. Hormones are chemically very diverse, including not only peptides encoded by genes, but also cholesterol derivatives (steroids) or amino acid derivatives, like the thyroid hormones (THs). Many animals have evolved endogenous hormone synthesis. As hormone action is dependent on the transduction of a signal initiated by direct binding to a receptor, hormone activity is tightly dependent on both upstream availability of hormones (production, transport, and degradation) and

downstream hormone signal transduction, initiated by a hormone binding to its receptor.

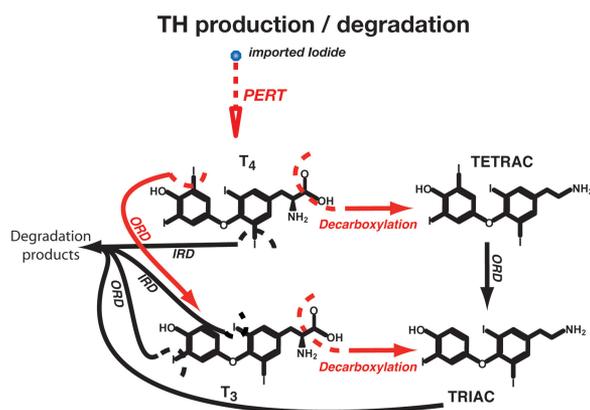
How the hormonal regulation of developmental processes evolved remains elusive, mostly because research has focused only on a few animal models (Miller and Heyland 2010). In the case of metamorphosis, although most animals metamorphose during their post-embryonic development (Nielsen 1998; Hadfield 2000), the molecular determinism of metamorphosis has been well studied only in some insects like the drosophilids, for which ecdysteroids and juvenile hormone are the main regulators, and in some vertebrates, like the amphibians, for which THs, and

especially the active TH  $T_3$  (3,3',5-triiodo-L-thyronine), are the main regulators (Leloup and Buscaglia 1977, for a general review, see Tata 2000). More precisely, production of the  $T_3$  precursor  $T_4$  (L-thyroxine), and to a lesser extent  $T_3$ , in the thyroid gland sharply increases at the onset of metamorphosis. In specific cells,  $T_4$  is metabolized into the more active TH  $T_3$ , among other TH derivatives (Fig. 1). Upon  $T_3$  binding, the thyroid hormone receptor (TR) initiates the modification of the transcription of target genes, eventually leading to the morphological remodeling characteristic of metamorphosis (Tata 2006).

THs were long believed to be vertebrate (and even gnathostome)-specific because only vertebrates had been clearly reported to (i) produce THs and (ii) metamorphose in a TH/TR-dependent manner (Nielsen 1998). However, TH production and an active TH metabolism, resembling the one in vertebrates, have been demonstrated outside vertebrates, and notably in the urochordate *Ciona intestinalis* (Barrington and Thorpe 1965; Patricolo et al. 2001; Shepherdley et al. 2004), in the cephalochordate amphioxus (Covelli et al. 1960; Tong et al. 1962; Monaco et al. 1981; Fredriksson et al. 1985) and even among non-chordates like in the sea urchin (Chino et al. 1994; Heyland et al. 2006). In addition, a few articles have demonstrated a link between TH metabolism and metamorphosis in non-vertebrates chordates and in echinoderms (Wickstead 1967; Chino et al. 1994; Patricolo et al. 2001; Heyland et al. 2006; Paris et al. 2008a). However, the

molecular mechanism behind TH action outside vertebrates is still unclear. In order to gain a better insight into the evolution of TH signaling and metamorphosis, we focused on the basal chordate amphioxus because comparisons between amphioxus and any other members of the chordate lineage, comprised of the vertebrates, the urochordates (like *C. intestinalis*) and the cephalochordates (like amphioxus), allows one to make inferences about the chordate ancestor (Delsuc et al. 2006). Using phylogenetic approaches on the recently sequenced amphioxus genome (Putnam et al. 2008; Holland et al. 2008a), we have shown that most of the genes involved in TH metabolism are present in amphioxus (Paris et al. 2008b), suggesting a deep ancestry of TH metabolism in chordates (Eales 1997). Moreover, we have shown that THs induce metamorphosis by activating the ortholog of the vertebrate receptors' TRs (Paris et al. 2008a). Interestingly,  $T_3$  acetic derivative 3,3',5-triiodothyroacetic acid (TRIAC), but not its precursor  $T_3$ , is a very potent inducer of metamorphosis and it binds TR, whereas  $T_3$  is a poor TR ligand (Paris et al. 2008a) as recently confirmed in another amphioxus species, *Branchiostoma belcheri* (Wang et al. 2009). Although  $T_3$  and  $T_4$  have been detected in amphioxus before (Covelli et al. 1960; Tong et al. 1962; Paris et al. 2008a; Wang et al. 2009), it was unknown whether TRIAC is naturally produced in amphioxus, as it is in vertebrates (Wu et al. 2005). The first part of this article demonstrates that amphioxus is able to metabolize  $T_4$  and  $T_3$  into their acetic derivatives tetraiodothyroacetic acid (TETRAC) and TRIAC, suggesting that these compounds can be endogenous ligands of the amphioxus TR *in vivo*.

More generally, although we had previously shown that THs induce metamorphosis in amphioxus, we had not investigated whether an active TH metabolism is responsible for triggering metamorphosis. Our previous phylogenetic analysis, as well as various other studies, had made us confident that TH synthesis is performed through a pathway similar to the vertebrates one (reviewed by Paris and Laudet 2008). In the second part of this article, in order to analyze the activity of TH metabolism during metamorphosis in amphioxus, we first tested whether compounds known to inhibit TH synthesis in vertebrates have an effect on metamorphosis. In addition, we measured the expression level of some interesting members of the TH-signaling pathway from fertilization to adult, with an emphasis on the time window when metamorphosis occurs. Taken together, these results suggest that TH metabolism is active during



**Fig. 1**  $T_4$  and  $T_3$  metabolism in amphioxus. TH metabolism is characterized by the fixation of exogenous iodine into  $T_4$  and  $T_3$  that are subsequently degraded into derivatives. Enzymatic steps that have been demonstrated in amphioxus are drawn in red. IRT: inner-ring deiodination; ORBD: outer-ring deiodination, PERT: thyroid peroxidase;  $T_3$ : 3,3',5-triiodo-L-thyronine;  $T_4$ : L-thyroxine; TRIAC: 3,3',5-triiodothyroacetic acid; TETRAC: tetraiodothyroacetic acid.

post-embryonic development and triggers metamorphosis in amphioxus.

## Materials and methods

### Chemical and reagents

L-[<sup>125</sup>I]-Thyroxine ([<sup>125</sup>I]-T<sub>4</sub>, radiochemical purity more than 95%) with specific activity of 4.29 TBq/mmol and L-[<sup>125</sup>I]-3,3',5-triiodo-L-thyronine ([<sup>125</sup>I]-T<sub>3</sub>, radiochemical purity more than 95%) with specific activity of 3.60 TBq/mmol were obtained from PerkinElmer Life Sciences (Courtabœuf, France). All solvents were of analytical grade and were purchased from Scharlau (Barcelona, Spain). Ultrapure water was produced by a Milli-Q water purification system (Millipore, Saint Quentin en Yvelines, France). The Extrelut<sup>®</sup> NT columns and solid phase for liquid–liquid extraction were purchased from Merck (VWR International, Fontenay-sous-Bois). T<sub>4</sub>, T<sub>3</sub>, 3,3',5'-triiodo-L-thyronine (reverse-T<sub>3</sub>), TRIAC, TETRAC, 3,5-diiodo-L-thyronine (3,5-T<sub>2</sub>), 3-iodo-L-tyrosine (MIT), diiodo-tyrosine (DIT), L-3,5,3'-Triiodothyronamine (T<sub>3</sub>AM), and 3,3',5-triiodothyropropionic acid (T<sub>3</sub>PA) standards were supplied from Sigma-Aldrich (Saint Quentin Fallavier, France).

Embryonic and larval culture, treatment with THs and monitoring of the advancement of metamorphosis

Embryonic cultures were done as previously described (Paris et al. 2008a). Advancement of metamorphosis was scored using morphological criteria as previously described (Paris et al. 2008a); briefly, metamorphosis is characterized by the symmetrization of a pelagic and highly asymmetric larva into a benthic and more symmetric juvenile.

Treatments with T<sub>3</sub>, TRIAC, propylthiouracil (PTU), thiouracil (TU), 2-mercapto-1-methylimidazole (MMI) and potassium perchlorate (KClO<sub>4</sub>) (Sigma, St. Louis, MO) were carried out as indicated in each experiment and are briefly described hereafter. In order to monitor the effects of blocking TH synthesis on metamorphosis advancement *in vivo*, we raised larvae together until the beginning of treatment. Then the larvae were separated in batches of about 50 (38–60) individuals for all treatments except for T<sub>3</sub> and TRIAC treatments for which only five individuals were used in both cases. The average metamorphosis advancement was calculated for each treatment each day. The effects of the different treatments on metamorphosis advancement were compared using a Kolmogorov–Smirnov test (Bonferroni-corrected for multiple comparisons with the control).

In order to measure gene expression throughout development, we raised larvae together for the time of the experiment. Fifty larvae were randomly sampled from the pool of animals at indicated time points (1, 3, 5, 9, 10 or 11 days after fertilization) or depending on their metamorphosis advancement (Pre-metamorphosis, when larvae have nine gill slits and harbor no sign of metamorphosis, metamorphosis stages M1–M5, individuals that were a few days older than M5, juveniles that were at least 2 months old). Material from TH treatment was described earlier (Paris et al. 2008a). Seawater complemented with the appropriate treatment was daily renewed in the cultures.

Treatment of adult amphioxus with radiolabeled THs

Adult *Branchiostoma lanceolatum* were collected in the bay of Argelès-sur-Mer (Mediterranean sea, France) as previously described (Fuentes et al. 2004). The animals were placed in 2.5-l plastic aquaria containing sand (depth of 3–5 cm) during two weeks for quarantine at 18°C. Four lots of 10 animals were then transferred into 200 ml of sea water filtered on a 0.22 μm filter. Substrates were incubated with amphioxus as follows: [<sup>125</sup>I]-T<sub>4</sub> (24 μCi, two concentrations: 10<sup>-7</sup> and 10<sup>-10</sup> M) and [<sup>125</sup>I]-T<sub>3</sub> (24 μCi, two concentrations: 10<sup>-7</sup> and 10<sup>-10</sup> M). Exposures were performed at 18°C for 48 h. Adults were sacrificed in liquid nitrogen and samples were kept frozen at -20°C until analysis. A similar treatment of 200 ml of water without animals was also performed for measuring natural degradation of THs. Radioactivity was quantified using a Gamma counter (Cobra, Packard, PerkinElmer Life Sciences). Iodine-1285 decay chart was applied to correct the amount of radioactivity.

Extraction of radiolabeled THs from treated adults

The adult samples (10 g) from adult amphioxus were mixed with 10 ml HCl 0.5 N and homogenized at room temperature using a Polytron homogenizer (Kinematica). The mixture was digested for 2 h in an oven at 100°C. The digest was then homogenized with 12 g of Extrelut NT solid phase and the Extrelut NT columns were filled. The radiolabeled compounds were eluted with 15 ml dichloromethane, then with 15 ml ethyl acetate.

Metabolic profiling

Identification of T<sub>3</sub> and T<sub>4</sub> metabolites was based on their retention times in two different HPLC systems as compared with those of authentic standards. The HPLC pump consisted of a model 980 solvent delivery (Jasco, Bouguenais, France) and a UV detector

model 975 (Jasco) set at 254 nm. A radiometric flow scintillation analyzer Flo-One  $\beta$  A250 (PerkinElmer) equipped with a solid detection cell was used for online detection of radioactivity.

Metabolites were quantified by integrating the area under the peaks detected by radioactivity.

### HPLC system #1

The HPLC analytical column was a reverse phase Zorbax Bonus RP (Agilent Technologies, Massy, France) using a binary step-gradient at 1 ml/min. The mobile phases contained 20 mM ammonium acetate buffer (pH 3) and acetonitrile in a combination of 90%/10% (mobile phase A) and 10%/90% (mobile phase B). Solvents were delivered as follows: 0–5 min, 70% mobile phase A; 5–20 min, linear gradient from 70% mobile phase A to 100% mobile phase B; 20–30 min, 100% mobile phase B. In this system, retention times were as follows:  $T_4$ : 11.80 min,  $T_3$ : 9.80 min, reverse- $T_3$ : 11.60 min, TRIAC: 20.30 min, TETRAC: 22.10 min, 3,5- $T_2$ : 5.50 min, MIT: 5.40 min, DIT: 3.80 min,  $T_3$ AM: 8.00 min, and  $T_3$ PA: 19.90 min.

Results presented here were obtained using this HPLC system.

### HPLC system #2

The HPLC analytical column was a reverse phase Kromasil  $C_{18}$  (Varian France, Les Ulis, France) using a binary step-gradient at 1 ml/min. The mobile phases contained 20 mM ammonium acetate buffer (pH 4.5) and acetonitrile in a combination of 90%/10% (mobile phase A) and 10%/90% (mobile phase B). Solvents were delivered as follows: 0–5 min, 70% mobile phase A; 5–25 min, linear gradient from 70% mobile phase A to 100% mobile phase B.

In this system, retention times were as follows:  $T_4$ : 12.10 min,  $T_3$ : 9.30 min, reverse- $T_3$ : 10.00 min, TRIAC: 15.50 min, TETRAC: 17.90 min, 3,5- $T_2$ : 4.50 min, MIT: 4.00 min, DIT: 3.40 min,  $T_3$ AM: 12.80 min, and  $T_3$ PA: 17.60 min.

Results obtained with this HPLC system were similar to the ones obtained with the HPLC system 1 and summarized in Fig. 2.

### Quantitative RT-PCR

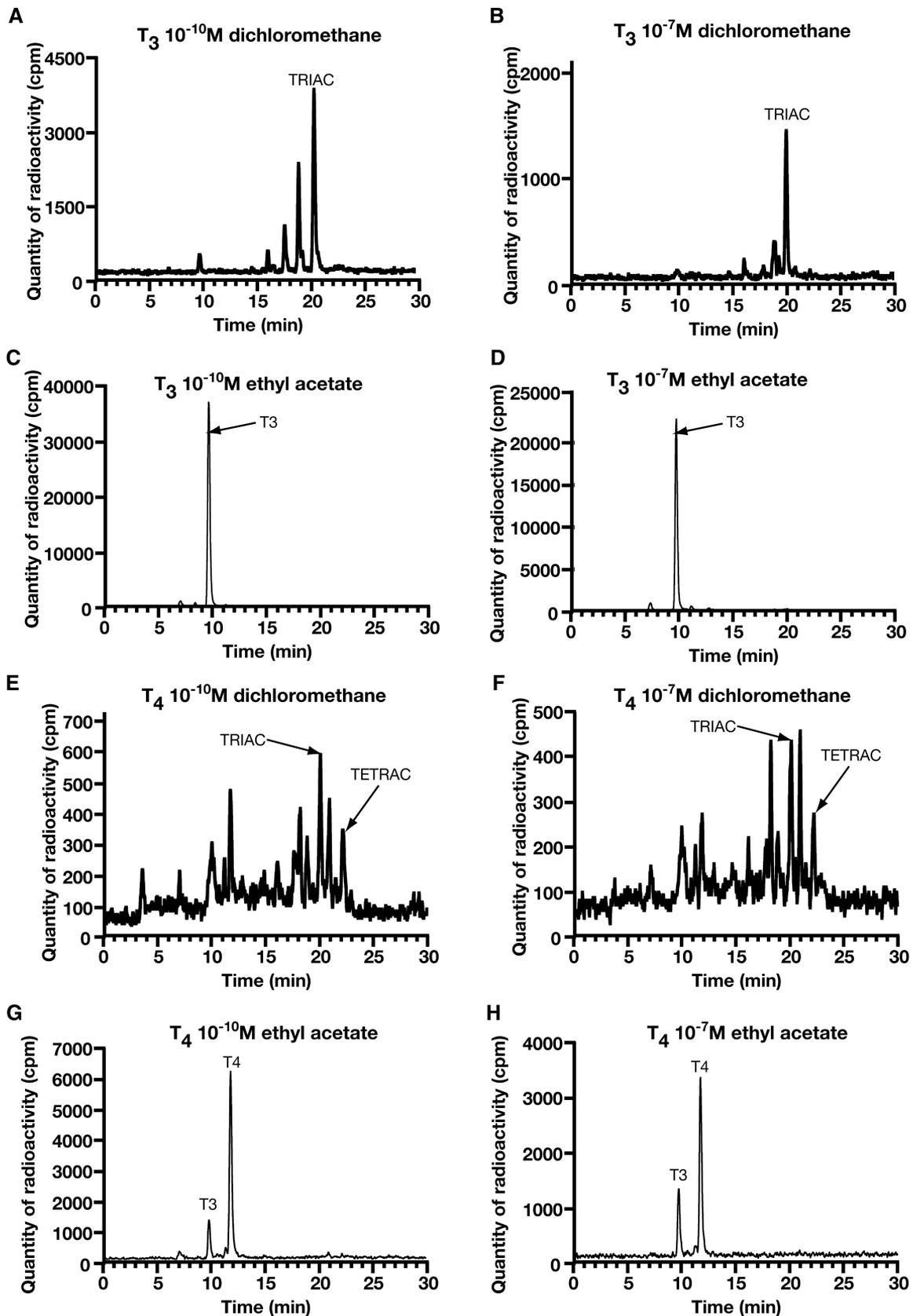
Total RNAs were prepared from pooled larvae (batches of 50 larvae for experiments describing gene expression during throughout development or batches of 10 larvae for TH treatment experiment) with Trizol (Invitrogen) according to the manufacturer's instructions and reverse-transcribed with random primers and MMLV Reverse Transcriptase

(Invitrogen). The cDNA was then used as a template for a quantitative real-time PCR assay with the QuantiTect SYBR Green PCR reagents (QIAGEN) and the DNA Engine Opticon system (MJ Research), as previously described (Paris et al. 2008a). Expressions of all genes were normalized to the corresponding 28S expression levels. All experiments were done two to five times in triplicates, except IOD $\epsilon$  expression in  $T_3$  treated/untreated larvae, which was measured once in triplicates. Error bars in Fig. 4 represent standard deviation. The sequences of forward and reverse primers were as follows: 28S-F (5'-ACTCTGGATAACCCAGCCGAT-3'), 28S-R (5'-TG CCTTCCTTGGATGTGGTAG-3'), amphitr-F (5'-CCGATGCATGACATGTGAAGG-3'), amphitr-R (5'-CACTTCTTGAAGCGCACTCC-3'), IOD $\epsilon$ -F (5'-GGCTGGTCCTTCAGAAACA-3'), and IOD $\epsilon$ -R (5'-ATAGCGGACCGGATATGTTG-3').

## Results

### $T_3$ , $T_4$ , and their acetic derivative production in amphioxus

We had previously made measurements of  $T_3$ ,  $T_4$ , TRIAC, and TETRAC by mass spectrometry on extracts from adults and were able to detect  $T_3$  at about 25 ng/g of wet mass in the amphioxus species *Branchiostoma floridae* (Paris et al. 2008a).  $T_3$  and  $T_4$  were also recently detected in another amphioxus species (Wang et al. 2009). However, neither TRIAC nor TETRAC could be detected. Interestingly, in vertebrates TRIAC and TETRAC are naturally produced from  $T_3$  and  $T_4$  but are rapidly degraded enzymatically and are thus kept at very low concentrations in the organism (Wu et al. 2005). In order to test whether TRIAC is experiencing the same fate in amphioxus, and to improve the sensitivity of detection in our experiments, we studied the metabolic fate of exogenous radiolabeled  $T_3$  and  $T_4$  in amphioxus. For that purpose, we treated batches of 10 adults with radiolabeled  $^{125}\text{I}-T_3$  and  $^{125}\text{I}-T_4$  at a concentration of  $10^{-10}$  M (Fig. 2A, C, E, and G). As the amount of radiolabeled TH easily handled is rather low [at least 1000 times lower than the dose administered to adults in previous studies (Paris et al. 2008a; Wang et al. 2009)], we also performed a second experiment, in which we tested the effect of  $10^{-7}$  M TH ( $^{125}\text{I}-T_3$  and  $^{125}\text{I}-T_4$  were adjusted to the appropriate concentration with unlabeled  $T_3$  and  $T_4$ , respectively, Fig. 2b, D, F, and H). The goal was to boost a possibly inactive pathway of TH signaling. After a 48 h treatment, the radiolabeled compounds were extracted from treated animals and separated by



**Fig. 2** TRIAC is a natural TH derivative in amphioxus. Amphioxus adults were treated with radiolabeled T<sub>3</sub> (A–D) and T<sub>4</sub> (E–H) for 48 h, and the radioactivity present in the animals was extracted by solid phase extraction and eluted by dichloromethane and ethyl acetate prior to radio-HPLC analysis. Dichloromethane extracts (A, B, E, and F) contain acetic derivatives of T<sub>3</sub> and T<sub>4</sub>, whereas T<sub>3</sub> and T<sub>4</sub> are present in ethyl acetate extracts (C, D, G, and H). Two doses were tested and gave similar results: 10<sup>-10</sup> M (left panels) and 10<sup>-7</sup> M (right panels). Metabolic profiles of dichloromethane extracts exhibited several unidentified metabolites. The results presented here were obtained with the HPLC system #1, as described in the Materials and methods section. Cpm: counts per minute.

high performance liquid chromatography (HPLC). Both concentrations ( $10^{-10}$  and  $10^{-7}$  M TH) gave very similar results (Fig. 2, left versus right panels).

As can be seen in Fig. 2, TRIAC was found to be a major metabolite of  $T_3$  (Fig. 2A and B), and TETRAC was also synthesized from  $T_4$ , although to a lesser extent (Table 1, Fig. 2E and F). In addition,  $T_3$  was also found to be a major  $T_4$  metabolite (Fig. 2G and H). Consistent with these findings, TRIAC was synthesized from  $T_4$  (Fig. 2E and F). Several chemical reactions may allow  $T_4$  to be transformed into TRIAC ( $T_4 \rightarrow$  TETRAC  $\rightarrow$  TRIAC or  $T_4 \rightarrow T_3 \rightarrow$  TRIAC; see Fig. 1), but our experimental procedure would not allow us to discriminate between them. The remaining peaks could not be assigned to any other TH derivative we tested, namely: 3,5- $T_2$ , reverse- $T_3$ , diiodotyrosine (DIT), monoiodotyrosine (MIT), 3,3',5-triiodothyropropionic acid ( $T_3$ PA) or the decarboxylated  $T_3$  derivative triiodothyronamine ( $TR_3$ AM) (data not shown). Whether other deiodinated acetic derivatives, like diiodothyroacetic acid, may correspond to these 'orphan' peaks will require further investigation. Incubations of  $^{125}$ I- $T_3$  and  $^{125}$ I- $T_4$  with amphioxus larvae were performed in the same experimental conditions as for adults (data not shown). Results were inconclusive in regards of biotransformation of  $T_3$  and  $T_4$  by amphioxus larvae because too little biological material was recovered. However, no TRIAC or TETRAC was detected demonstrating that TRIAC and TETRAC cannot be considered as a result of the extraction procedure or as degradation products. Overall, the experiments described in Fig. 2 demonstrate that TRIAC and TETRAC are major natural derivatives of  $T_3$  and  $T_4$  in adult amphioxus.

### Blocking production of TH inhibits metamorphosis in amphioxus

To confirm that THs are not only sufficient (Paris et al. 2008a) but also necessary for triggering

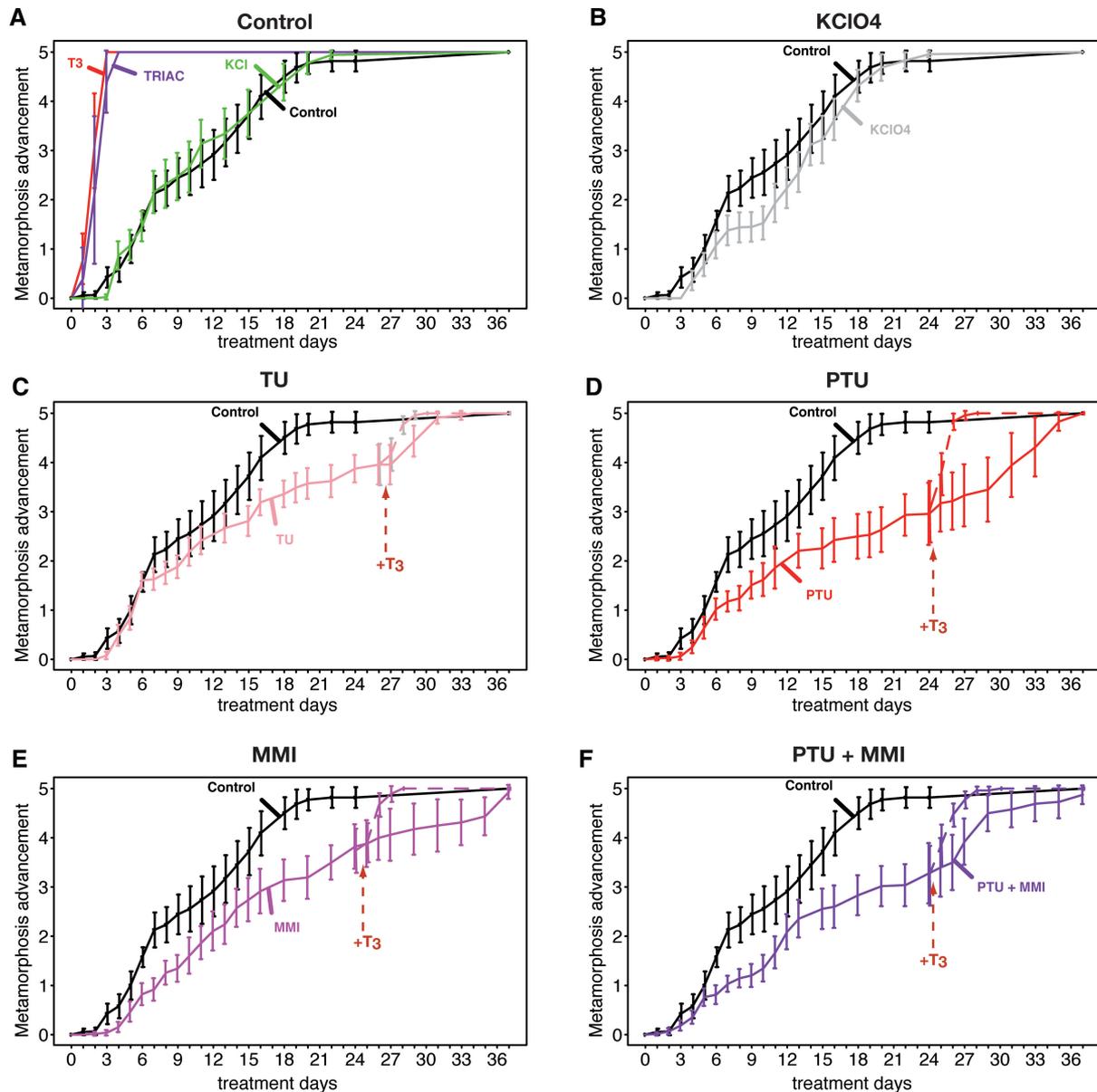
**Table 1** Recovery of TRIAC and TETRAC in dichloromethane extracts of amphioxus treated with  $T_3$  ( $n = 3$ ) and  $T_4$  ( $n = 4$ ) expressed as percentage of the total radioactivity (mean  $\pm$  SD). TETRAC was undetectable in the  $T_3$ -treated samples ("").

Treatment	Dichloromethane extraction	
	% TRIAC	% TETRAC
$T_3$ ( $10^{-7}$ M)	1.9 $\pm$ 0.4	/
$T_3$ ( $10^{-10}$ M)	1.7 $\pm$ 0.2	/
$T_4$ ( $10^{-7}$ M)	0.8 $\pm$ 0.5	0.5 $\pm$ 0.4
$T_4$ ( $10^{-10}$ M)	0.3 $\pm$ 0.2	0.4 $\pm$ 0.4

metamorphosis, we tested the effects of blocking TH synthesis on amphioxus' metamorphosis. For that purpose, we treated batches of 11-day-old pre-metamorphic larvae with various compounds (collectively referred as 'goitrogens') known to interfere with TH metabolism in vertebrates, and monitored the effect of these compounds on the advancement of metamorphosis. We selected this approach because several analyses of various kinds (phylogenomic, developmental, biochemical) have suggested that the production of TH occurs in similar ways in amphioxus and in vertebrates (e.g., Monaco 1981; Ogasawara 2000; Paris et al. 2008b; reviewed by Paris and Laudet 2008). For instance, peroxidase-dependent iodine fixation and TH production have been previously reported in amphioxus (Tong et al. 1962; Monaco et al. 1981) and we have demonstrated deiodinase activity in amphioxus (Paris et al. 2008a). In addition homologues to thyroid peroxidase (PERT), iodine transporter SIS and deiodinases, involved in the production of TH in vertebrates, have been found in the amphioxus genome (Paris et al. 2008b). We tested the effect of (i) TU, (ii) MMI that both interfere with PERT function, (iii) PTU that inhibits both PERT and  $T_4$  to  $T_3$  deiodination, and (iv)  $KClO_4$ , an anionic competitor of iodine uptake by SIS (these goitrogens, were previously shown to inhibit metamorphosis in the basally divergent vertebrate lamprey) (Manzon et al. 2001). KCl was used as a negative control and  $T_3$  and TRIAC were used as positive controls of metamorphic competence.

As seen in Fig. 3, the goitrogens inhibit metamorphosis with various efficiencies (Fig. 3B–F): PTU, MMI, or the combination of both, significantly lowered the average daily metamorphosis score compared to the control ( $P$ -values  $< 0.05$ , Fig. 3D, E, and F). However, the negative control KCl, as well as  $KClO_4$  and TU had not significant effects, compared to the control (Fig. 3A, B, and C). On the opposite, the two positive controls  $T_3$  and TRIAC strongly induced metamorphosis (Fig. 3A). Interestingly, exogenous  $T_3$  significantly recovered advancement of metamorphosis in animals treated with PTU, MMI or both (dashed lines in Fig. 2D–F), suggesting that inhibitory effects of these goitrogens are in a large part due to deficiency in TH production.

The inhibition of metamorphosis by goitrogens was only partial, since treated animals eventually metamorphosed. The experiment described here was started shortly before natural metamorphosis occurred (metamorphosis started at Day 2 in the control). It is thus possible that goitrogenic action was



**Fig. 3** Blocking endogenous TH production inhibits metamorphosis of amphioxus. Effects on metamorphosis of batches of 38–60 11-day-old premetamorphic amphioxus larvae of  $T_3$  at  $10^{-8}$  M, TRIAC at  $10^{-8}$  M or KCl at  $5 \times 10^{-4}$  M (A),  $KClO_4$  at  $5 \times 10^{-4}$  M (B), TU at  $5 \times 10^{-4}$  M (C), PTU at  $5 \times 10^{-4}$  M (D), MMI at  $5 \times 10^{-4}$  M (E) or a mix of PTU and MMI at  $5 \times 10^{-4}$  M each (F) were monitored using five specific morphological criteria [fully described by Paris et al. (2008a)]. (C–F) After 25 days of treatment, batches were split in two and exogenous  $T_3$  at  $10^{-8}$  M was added to one half of the larvae (dashed lines). Error bars correspond to a 5% confidence interval. PTU, MMI or the combination of both have a significant effect on metamorphosis ( $P$ -value  $< 0.05$ ) and exogenous  $T_3$  significantly counteracts these actions.

initiated too late for a complete disruption of TH production (e.g., TH levels increase before amphibian metamorphosis starts [Tata 2006] and we may have the same phenomenon in amphioxus). To tackle this issue, we repeated the experiment with younger larvae and started treatment with goitrogens four days after fertilization (Supplementary Fig. 1): metamorphosis was inhibited with a better efficiency (especially by the combination of MMI and  $KClO_4$ ,

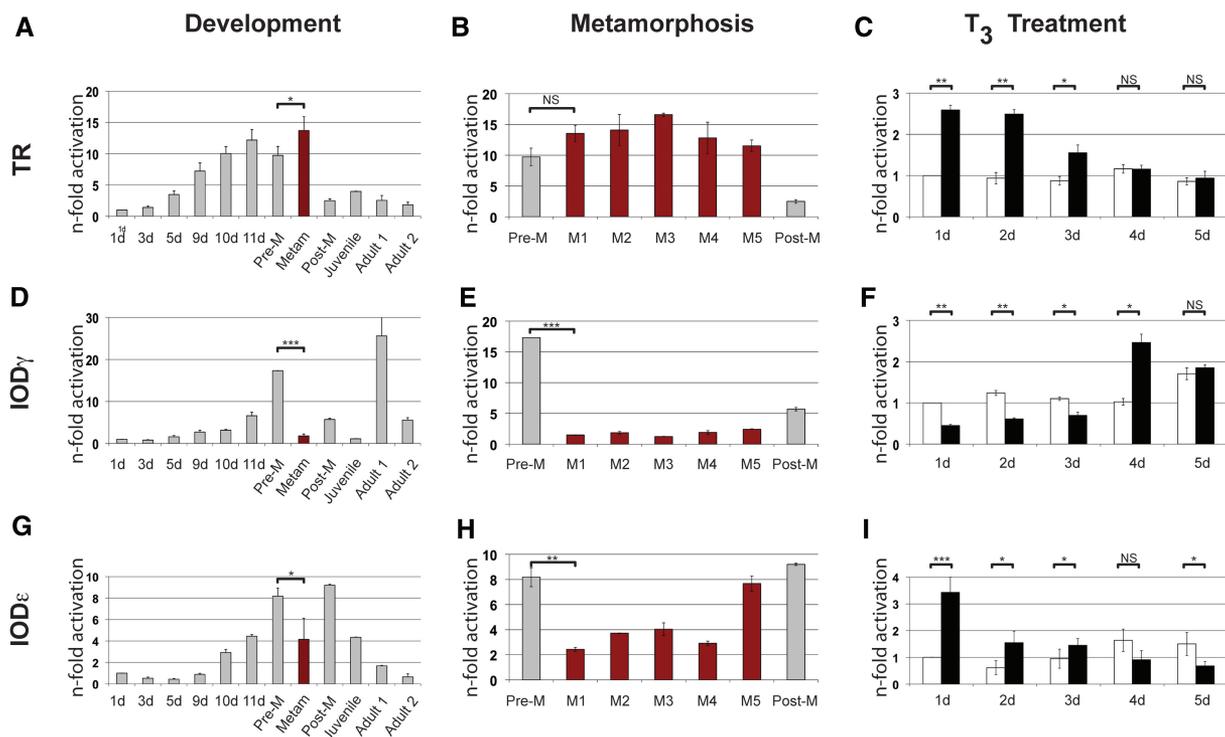
that was not tested the first time, Supplementary Fig. 1F), but was not completely blocked, suggesting that (i) THs are not the only triggering factor, (ii) goitrogens do not accumulate well in amphioxus tissues or are rapidly degraded, or (iii) goitrogens do not block functioning of PERT, deiodinase, or SIS (transporter responsible for accumulation of iodine) as well as in vertebrates. It is difficult to decide which hypothesis should be favored without further

biochemical experiments on PERT, deiodinase, and SIS. However, and this is of special interest here, goitrogens inhibit metamorphosis, thus confirming the important role of THs as regulators of this process in amphioxus. Of note, onset of metamorphosis was not delayed, since treated animals entered metamorphosis the same day as did controls. This suggests that treatment with goitrogen inhibited later stages rather than earlier stages of metamorphosis.

### TH metabolic pathway during development in general and through metamorphosis in particular

We monitored the expression of members of the TH signaling pathway (TR, two different deiodinase homologues IOD $\gamma$  and IOD $\epsilon$  that were likely to be functional [Paris et al. 2008b] and two PERT homologs) throughout amphioxus' development, from fertilization to adult (Fig. 4 and data not shown). Special attention was paid to metamorphosis, for which data on five distinct stages were collected.

TR displayed the most compelling profile: its expression increased early and steadily until it peaked right at metamorphosis (Fig. 4A and B). In addition, TR expression was enhanced by T<sub>3</sub> (Fig. 4C), as previously suggested (Paris et al. 2008a). This expression profile is similar to TR $\beta$  expression during amphibian metamorphosis, in which TR $\beta$  plays a central role as T<sub>3</sub> receptor. The expression of IOD $\gamma$  increased throughout development until it sharply dropped at the onset of metamorphosis (more than 15-fold reduction of expression between premetamorphic stage and first metamorphic stage M1, Fig. 4E). Its expression remained low throughout metamorphosis and increased only when metamorphosis was complete (Fig. 4D and E). T<sub>3</sub> treatments on premetamorphic larvae first mildly repressed its expression and then induced it after several days of treatment, when larvae were fully metamorphosed (Fig. 4F). IOD $\epsilon$  expression also increased until metamorphosis, then decreased about three times at the onset of metamorphosis but



**Fig. 4** The expression of TH signaling genes is correlated with metamorphosis and is modulated by exogenous THs. The expression of amphitr (A–C), IOD $\gamma$  (D–F), and IOD $\epsilon$  (G–I) in amphioxus was measured by quantitative RT-PCR during development from fertilization to adult (A, B, D, E, G, and H), with an emphasis on metamorphosis (B, E, and H). Metamorphosis is represented by a unique value in (A, D, and G) and corresponds to the mean of five different values throughout metamorphosis in (B, E, and H). The effect of T<sub>3</sub> at 10<sup>-8</sup> M on gene expression was also monitored during 5 days in 18-day-old premetamorphic larvae (C, F, and I). Panel (C) confirms previously reported results (Paris et al. 2008a). Measurements were made on RNA extracted from pools of 50 embryos for each time points of (A, B, D, E, G, and H) and 10 embryos otherwise (C, F, and I). Expression values are displayed relative to 1-day-old embryos in (A, B, D, E, G, and H), and untreated animals from the first day of treatment in (C, F, and I). Error bars represent standard deviation. Student's *t*-test: \**P*-value < 0.05; \*\**P*-value < 0.01; \*\*\**P*-value < 0.001; NS: *P*-value > 0.05.

increased again before the end of metamorphosis (Fig. 4G and H). In addition, T<sub>3</sub> treatments on pre-metamorphic larvae quickly enhanced IOD $\epsilon$  expression (Fig. 4I).

None of the two thyroid peroxidase homologues, TPO515 and TPO517 we studied, exhibited an expression profile obviously related to metamorphosis (data not shown).

## Discussion

We had previously shown that TRIAC, the acetic derivative of the TH, T<sub>3</sub>, potently induces metamorphosis in amphioxus through a unique TR ortholog that mediates its action. This assertion was based on three observations: TRIAC is a potent inducer of metamorphosis, it is a high-affinity TR ligand, unlike T<sub>3</sub>, and blocking the action of TR inhibits TRIAC effects on metamorphosis (Paris et al. 2008a). However, we had not investigated whether TRIAC could be the actual active compound during metamorphosis. Here we show that TRIAC is naturally produced by amphioxus adults. More generally, we report *in vivo* and *in vitro* assays that support the presence of an active metabolism of TH during metamorphosis of amphioxus.

### TH repertoire of amphioxus resembles, but is not identical with, the vertebrate repertoire

To our knowledge, only a 50-year-old study describes the presence of TRIAC in amphioxus using radiolabeled iodine (Covelli et al. 1960) and we had previously found no TRIAC in amphioxus using direct measurements (data not shown in Paris et al. 2008a). This failure was questioning whether TRIAC is a natural TH compound in amphioxus. Here we show that amphioxus is able to transform T<sub>3</sub> into TRIAC and T<sub>4</sub> into T<sub>3</sub>, TETRAC and TRIAC, thus demonstrating that TRIAC is a natural compound in amphioxus, as in vertebrates, and that amphioxus possesses an enzyme able to perform the oxidative deamination necessary for the transformation of T<sub>3</sub> into TRIAC and of T<sub>4</sub> into TETRAC (Fig. 1).

Our experiments also reveal differences in TH metabolism of amphioxus compared to that of vertebrates. Indeed, reverse-T<sub>3</sub> appears to be produced in greater amounts in mammals than in amphioxus in which it was undetectable. Moreover, TETRAC is more abundant than TRIAC in mammals (Braverman and Utiger 1996), whereas it is the other way around in amphioxus. Other T<sub>3</sub> and T<sub>4</sub> metabolites could not be identified. Since the retention times of the major unidentified metabolites are

higher than those of available standards and are close to those of TRIAC and TETRAC (Fig. 2A, B, E, and F), it can be speculated that some of the derivatives of T<sub>3</sub> or T<sub>4</sub> could be further deiodinated, leading to di-iodo acetic derivatives, as described in vertebrates (Rutgers et al. 1989; Frith and Eales, 1996). Of note, we could not determine whether sulfation and glucuronidation are major pathways for degradation of TH, as in vertebrates (Moreno et al. 1994), because the acidic conditions under which THs were manipulated led to the hydrolysis of any potential glucuroconjugate and sulfoconjugate.

From this experiment, we can conclude that (i) TRIAC is a major derivative of amphioxus' TH metabolism, which strengthens its possible role during the metamorphosis of amphioxus (Paris et al. 2008a), and (ii) although very close, amphioxus' TH metabolism probably displays some clade peculiarities.

### Peak of TH production at metamorphic climax

Metamorphosis is triggered by exogenous THs, whereas it is inhibited by blocking endogenous TH production (Paris et al. 2008a and the data presented here). We conclude from these results that THs are key regulators of metamorphosis in amphioxus, as it is in amphibians, pushing the origin of TH-induced metamorphosis back to chordate ancestry. Interestingly, late rather than early metamorphic events were delayed by blocking production of TH (e.g., animals were simultaneously entering metamorphosis in control and treated animals, Fig. 3). These results may be paralleled with a previous experiment in which blocking TH receptor with a specific TR antagonist inhibited late, rather than early, metamorphosis (metamorphic stages 2 and later, Paris et al. 2008a). This suggests that the different events of morphological remodeling characteristic of metamorphosis are regulated by different levels of TH, with later stages regulated by high TH levels, as in amphibians (Tata 2000). Interestingly, treatments with THs of weaker biological effect (like reverse-T<sub>3</sub>) induce a desynchronized metamorphosis in amphioxus, with for instance morphological criteria of metamorphic stage 4 appearing before those of stages 2 and 3 (unpublished data). This is in agreement with tissue-specific remodeling events during metamorphosis, like cell migration in the gill slits, cell death in the club-shaped gland or in the mouth, cell proliferation in the cirri surrounding the mouth (Holland et al. 2008b); different tissues do not respond the same way to THs. Overall, our

results suggest cell-specific or organ-specific sensitivity to THs in amphioxus, allowing cell-specific and organ-specific responses to a general TH signal. Alternatively, a TH-independent pathway may be involved in the regulation of earlier metamorphic events. In this case, however, this pathway would be connected to the TH-pathway since THs are sufficient to trigger metamorphosis (Paris et al. 2008a). Overall, we propose that there is an active TH signaling pathway that regulates metamorphosis in amphioxus larvae (Paris and Laudet 2008).

### Expression of members of the TH metabolic pathway during metamorphosis

We studied the expression of several amphioxus genes homologous to vertebrate members of the TH metabolic pathway during development, with an emphasis on metamorphosis. We also measured the effect of  $T_3$  on the expression of these genes in premetamorphic larvae. More precisely, TR, two deiodinases and two peroxidases were included in this assay. As previously proposed, TR expression peaks at metamorphosis (Fig. 4A and B), as in amphibians (Tata 2000).

Previous sequence analysis of IOD $\gamma$  and IOD $\epsilon$  had shown that both genes encode potential functional deiodinases, but did not give insight into specificities of the proteins regarding the TH ligand (Paris et al. 2008b). Here we show that gene expression correlated with metamorphosis with high expression before or after metamorphosis and low expression during metamorphosis. However,  $T_3$  rapidly repressed IOD $\gamma$  expression in premetamorphic larvae and induced its expression only when larvae had metamorphosed when it had the opposite effect on IOD $\epsilon$ . Overall, these results suggest that IOD $\gamma$  and IOD $\epsilon$  are involved in metamorphosis, probably as deiodinases, but do not respond similarly to high TH levels, and may thus have different roles in the regulation of TH degradation. Further experiments will be required to prove the involvement of these proteins in metamorphosis regulation (see below).

The presence of active deiodinases is supported by the HPLC data (Fig. 2) that suggest that there are active outer-ring deiodinases in amphioxus, since  $T_3$  and TRIAC can be produced from  $T_4$  (Fig. 1 and 2E–H), confirming the results of previous experiments based on *in vivo* treatments with a deiodinase inhibitor (Paris et al. 2008a; Wang et al. 2009). Indeed, transformations of  $T_4$  to  $T_3$  and of TETRAC to TRIAC are realized by removing one iodine from the outer ring of  $T_4$  and TETRAC (Fig. 1). At least some of amphioxus' genes

homologous to vertebrates' deiodinases, among which IOD $\gamma$  and IOD $\epsilon$  (Paris et al. 2008b), are probably responsible for this deiodinase activity. Outer-ring deiodination, also evidenced in urochordates (Shepherdley et al. 2004), then seems to be ancestral in chordates. Whether amphioxus may be able to perform inner-ring deiodination remains to be established, possibly by identifying the extra peaks in Fig. 2. In addition, further biochemical analyses will be required in order to characterize the properties of the different proteins encoded by deiodinase homologues in amphioxus. In particular, *in situ* hybridization during metamorphosis would allow one to determine whether IOD $\gamma$  and IOD $\epsilon$  are specifically expressed in tissues subject to remodeling.

As goitrogens that are known to inhibit the action of PERT also inhibit spontaneous metamorphosis (Fig. 3), we expected amphioxus homologs to PERT to be highly expressed during metamorphosis. However, our data on the expression of peroxidase homologs do not allow us to confirm this hypothesis. Whether amphioxus' peroxidases have a role in TH metabolism will require additional experiments, and in particular *in situ* hybridization in metamorphic larvae, to see if one of the genes is expressed in the endostyle, the organ where THs are produced. Nonetheless, the described experiments on gene expression support the view that there is an active metabolism of TH during metamorphosis in amphioxus.

The role of TRIAC, and not  $T_3$ , as the potential thyroactive compound in amphioxus can be discussed in the context of the evolution of the TH signaling pathway. Our data have strengthened the previously formulated hypothesis that endogenous TH production predates the origin of chordates (Fredriksson 1985; Eales 1997; Ogasawara 2000). In addition, TH effects have been proposed to precede TH production in deuterostomes (Eales 1997; Heyland et al. 2006; Miller and Heyland 2010). According to this scenario, the source of TH was first exogenous and became internalized later during evolution. In vertebrates, THs are centrally produced mostly as the inactive form  $T_4$ , which is then transported to peripheral tissues where it is transformed into the active derivative  $T_3$ , in a cell-specific manner. It is tempting to speculate that such TH action, with a precursor centrally produced and specifically degraded into an active form, is common in chordates. In this case, an exogenous source of active TH may compete with the cell-specific control of TH metabolism. As phytoplanktonic organisms are able to produce  $T_3$  and  $T_4$  (Chino et al. 1994; Eales 1997; Heyland and

Moroz 2005), it is probable, although still speculative, that amphioxus and all planktonic marine chordates are exposed to these THs from the environment. Following the hypothesis that early chordates had a double  $T_3$  source, the degradation of an otherwise ancestrally inactive  $T_3$  into a more active derivative like TRIAC may be seen as an endogenous regulation of the exogenous source of TH. Following this scenario, the TH receptor TR would then be responsive only to active derivatives of  $T_3$ , which would allow an internal and cell-specific control upon both the exogenous and endogenous signals. This additional regulatory system could have persisted in some marine chordates like amphioxus. In non-marine chordates like mammals or amphibians, for which exogenous  $T_3$  sources are very scarce, selection pressure to keep the additional regulatory step would have weakened to the point where the previously inactive precursor  $T_3$  became the active molecule and only  $T_4$  kept the function of the ancestral precursor. Vertebrate TR strongly binding TRIAC could then be seen as a 'remnant' of the ancestral key role of TRIAC as the biologically informative molecule. Investigating the role of TRIAC and other  $T_3$  and  $T_4$  derivatives in invertebrate chordates and in echinoderms should enhance discussions of this hypothesis. The characterization of the echinoderm TR homolog (Howard-Ashby et al. 2006) and the analysis of the TH metabolism in other chordates, like lamprey or urochordates, will allow us to better understand the evolution of TH metabolism in chordates. More generally, our hypothesis calls for a less vertebrate-centered approach on the evolution of endocrine systems (Markov et al. 2008).

## Supplementary Data

Supplementary data are available at *ICB* online

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