Origin of thyroid hormone signalling in metazoans and implications in their metamorphosis

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Abstract

Metamorphosis is a critical life step of many metazoans. It is defined as the transition between a larva and a juvenile. It comes with major changes of morphology, physiology and ecology. The role of thyroid hormone has been proven in the metamorphosis of amphibians but also in many other chordates such as teleost fishes or the amphioxus. This suggests that this system was functional and involved in the metamorphosis of the common ancestor of all chordates.

We studied the link between thyroid hormones and metamorphosis according to two axes. First, we studied the coordination between metamorphosis and the larval colonization, using the convicted surgeon fish *Acanthurus triostegus* as a model. This work allowed us to better understand the role of thyroid hormones as a trigger signal of a major ecological transition. On the second axis, we investigated the origin of thyroid hormone signalization. In the annelid *Platynereis dumerilii*, we identified a functional thyroid hormone receptor and a role of TH in its development. This proves that thyroid hormone signaling was present at the basis of bilaterians. We also assessed the question of the origin of thyroid hormone synthesis by tracing back the evolution of the thyroglobulin, the protein mandatory for thyroid hormone synthesis in vertebrates. It absence in the other taxa, whereas thyroid hormone derivatives have a biological role, asks the question of the ancestral mechanisms of thyroid hormone synthesis.

These works investigate the evolution of the thyroid hormone signalization and suggest to tackle the question of the link with metamorphosis from an evolutionary perspective, and not only from a developmental one, in order to better understand the diversity of metamorphosis overserved in the animal kingdom.

Résumé

La métamorphose est une étape cruciale du cycle de vie de beaucoup de métazoaires. Elle est définie comme la transition d'une larve en un juvénile, souvent accompagnée de changements drastiques de morphologie, physiologie et de niche écologique. Le rôle des hormones thyroïdiennes a été mis en évidence dans la métamorphose des amphibiens, mais aussi de nombreux autres chordés comme certains poissons téléostéens ou l'amphioxus, suggérant que ce système était déjà présent et impliqué dans la métamorphose chez l'ancêtre commun de tous les chordés.

Nous avons étudié le lien entre hormones thyroïdiennes et métamorphose selon deux axes. Premièrement, nous avons étudié la coordination entre la métamorphose et le recrutement larvaire, chez le chirurgien bagnard *Acanthurus triostegus*. Ce travail nous a permis de mieux comprendre le rôle de l'hormone thyroïdienne comme signal déclencheur d'une transition écologique importante. Dans le second axe de travail nous avons examiné l'origine de la signalisation thyroïdienne. Chez l'annélide *Platynereis dumerili,* nous avons identifié un récepteur fonctionnel des hormones et un rôle de l'hormone thyroïdienne dans son développement. Cela qui nous permet de démontrer que la signalisation thyroïdienne était présente à l'origine des bilateriens. Nous avons également mis en évidence un rôle des hormones thyroïdiennes dans le développement de cette espèce. Enfin dans le troisième axe nous nous sommes penchés sur l'origine de la synthèse de l'hormone thyroïdienne en retraçant l'évolution de la thyroglobuline, la protéine nécessaire à la production d'hormone chez les vertébrés. Son absence dans les autres taxons, alors que les dérivés de l'hormone y ont un rôle biologique, pose la question des mécanismes ancestraux de synthèse de ces hormones.

Ces travaux explorent l'évolution de la signalisation thyroïdienne et proposent d'aborder la question du lien avec la métamorphose d'un point de vue évolutif et non-seulement développemental, afin de mieux comprendre la diversité des métamorphoses observées dans le monde animal.

Acknowledgments / Remerciements

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Enfin, un immense merci à ma famille qui m'a toujours soutenu.

Acronyms

3,3'-T2: 3,3'-diiodothyronine

3,5-T2: 3,5-diiodothyronine

DBD: DNA binding domain

CRF: Corticotropin-releasing factor

GC: Glucocorticoid

HPA: Hypothalamus-Pituitary-Adrenal

HPT: Hypothalamus-Pituitary-Thyroid

HRE: Hormone response element

LBD: Ligand binding domain

RXR: Retinoid X receptor

Tetrac: Tetraiodothyroacetic acid

Tg: Thyroglobulin

TH: Thyroid homrone

TPO: Thyroid peroxidase

TR: thyroid hormone receptor

TRE: Thyroid response element

TRH: Thyrotropin-releasing hormone

TRHR Thyrotropin-releasing hormone receptor

Triac: 3,5,3'-Triiodothyroacetic Acid

TSH: Thyroid stimulating hormone

TSHR: Thyroid stimulating hormone receptor

TTF-1: Thyroid transcription factor 1

T4: thyroxine

T3: triiodothyronine / 3,3',5-triiodo-L-thyronine

A beginning is a very delicate time

Contents

Organization of the manuscript

Metamorphosis is a fascinating process and an irreversible turning point of the life history of an individual with ecological, behavioural, morphological and physiological changes happening in a short period. Moreover, a lot of species undergo a metamorphosis during their life cycle. Thus, it is not only a subject of investigation from a developmental perspective, but it can also be approached by many other fields of biology, including evolutionary developmental biology.

The first physiological understanding of this phenomenon traces back to the early 20th century. From this starting point, the fundamental role of thyroid hormone has been investigated, proven and deciphered in many species. Most of our understanding of vertebrate metamorphosis comes from anourans with a biphasic life cycle (tadpole/frog), the role of thyroid hormone in metamorphosis has also been demonstrated in teleost fishes and the basal chordate amphioxus. My thesis engraves in this framework, the better understanding of the link between metamorphosis and thyroid hormone signalling among metazoan. The famous sentence (maybe too much quoted) of Theodosius Dobzhansky "*Nothing in biology makes sense except in the light of evolution*" gives the perspective by which I tried to tackle my thesis.

This work can be divided in two main axes investigated by three questions. On the first axis, we investigated the role thyroid hormone in the metamorphosis of the convicted surgeonfish *Acanthurus triostegus*. Given the peculiar life cycle of the coral reef fishes this work gives some insight on the coordination between the developmental event of the metamorphosis and the ecological event of the reef colonization. On the second axis, we investigated the evolution of the thyroid hormone pathway with two approaches. First we investigated the function of thyroid hormone in the annelid *Platynereis dumerilii*. With this worm we can improve our knowledge of the thyroid hormone signalling at the scale of metazoans. Second we investigated the sequence evolution of the thyroglobulin, the protein from which thyroid hormone is synthetized. This is to better understand the evolution of the hormone synthesis.

To introduce this work, I will give the broad context of evolutionary-developmental biology and the question it address. Then, I will present the thyroid hormone, its receptors and some actors of the thyroid hormone pathway since they are at the centre of my biological questions. Afterwards, I will present our current understanding of metamorphosis. Fourth, I will address the biological questions of my thesis and the model to answer them. After the introduction, the result will be presented in three drafts of publications and discussed to give a unifying perspective to this thesis. Additional publications are presented in the appendix.

I Introduction

1. Forewords on evolutionary developmental biology

The diversity of living species is astonishing, (Figure 1) in term of size and shape, as between a sea urchin and a blue whale. It is also in term of behaviour, ecology and metabolism. Plants can fix carbon from the atmosphere where animals must extract carbon form highly complex organic molecules. The evolutionary developmental biology (evo devo) investigates how developmental processes and mechanisms contribute to the evolution of species and the biodiversity we observe today

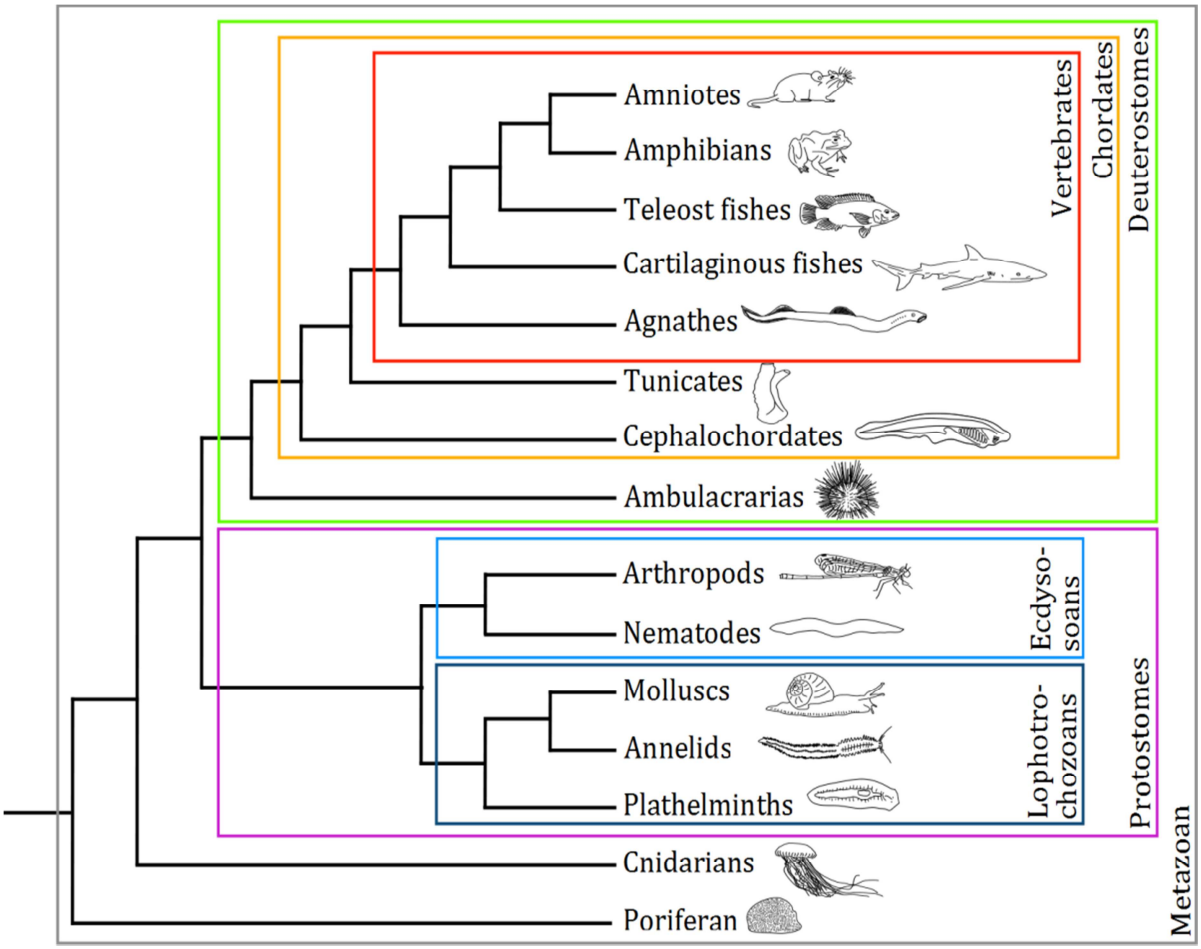

Figure 1: Simplified tree of metazoans. The main taxa that will be discussed appear in the rectangle boxes.

1.1 The scientific bloom of the 19th century

1.1.1. A brief history of the theory of evolution

The huge diversity of life forms was not unknown to the philosophers of the antique world and the idea of classification of living beings traced back to the first observations of the living world. Aristotle, in the antique Greece, proposed a classification and hierarchy of the animals (Lloyd, 1961) Much later, in the 18th century the Swedish naturalist Carl Linnaeus performed a colossal work of species identification and proposes a classification of plants and animals (Cain, 1958), creating the system of species naming that we still used today. Those classifications and all of them in between were mainly fixists, that is to say that species were *created* or *appeared*, as they are and there was no concept of change or evolution.

The French naturalist Jean-Baptiste de Lamarck was one of the first to popularize the idea that a species can change, in opposition to the fixism. Nevertheless his hypothesis of the transmission of acquired characteristics did not convinced and, aside for the nowadays concept of epigenetic, it happened to be wrong. It is 1859, in the middle of the effervescent 19th century, that the English naturalist Charles Darwin published his major book *The origins of species*, that formulates the still up to date theory of evolution (Darwin, 1859). In this work, Darwin proposed a complete intellectual framework to understand evolution. He developed the idea of offspring surplus, natural variation of characteristics and environmental selection upon those characteristics. As a consequence, he proposed that all animal species are related one to another and share a common ancestor. This idea was then expanded with input from other disciplines such as ecology, paleontology, genetic or population genetic. Around the 1940s, authors such as Ernst Mayr (Mayr, 1942), Theodosious Dobzhansky (Dobzhansky, 1937) , George Simpson (Simpson, 1944) and Julian Huxley (Huxley, 1942) integrate several fields of biology together and forge the synthetic theory of evolution. Nevertheless, development was not integrated yet.

1.1.2. A brief history of genetics

If today, DNA has become a common name that everyone knows, it has been a long road to understand that it is the molecule that carries genetic information. There was an empirical understanding of the transmission of characters since the beginning of the agriculture, as seeds or cattle were inbreed to safeguard and combine in offspring the characteristic of the parents. Nevertheless, the Austro-Hungarian monk Gregor Johann Mendel was the first to understand and describe the rules of the characteristic transmission between generations (Mendel, 1866). The modern genetics took a great leap with the concept of gene developed by Thomas Morgan (Morgan, 1926) , even if the support of the genetic information that is to say the entity that physically carries this information was unknown. Many investigations were performed to understand the molecular nature of this genetic information. One milestone was reached in 1944 when chromosomes were proven as the molecular carrier of the genetic information (Avery et al., 1944). Then, in 1953, Francis Crick and James Watson, with the help of Rosalind Franklin, discovered the molecular structure of DNA (Watson and Crick, 1953). The discovery of the codon as a unit of information by Francis Harry Compton Crick and colleagues in 1961 (Crick et al., 1961) and the codon/amino acid relationship by several independent teams open the way to the genetic code as we know it with the notion of mutation, silencing, gene regulation.

1.1.3. A brief history of the study of development

The study of development aims to understand the processes between the reproduction and the birth or adulthood of an individual. This is also a long standing subject of philosopher and naturalist. In the antiquity two hypotheses had emerged. The preformationism supported that individuals were already formed in the gamete of their parents, as tiny version of the adult, like Russian dolls, and called animalcule or homunculus for humans. In opposition, the epigenesis supported that the individuals and the organs are formed gradually. Many debates opposed the two hypotheses in the 18th century and early 19th century. In the beginning of the 19th century, the preformationist hypothesis will be definitively dismissed with the concept of the germ layer proposed by Caspar Wolff, Heinz Pander and Karl von Baer (Aulie, 1961; Kain et al., 2014; Wolff, 1759) and the description of the mammalian ovule by Karl von Baer in 1827 (von Baer, 1828; Lopata, 2009). This paved the way to the modern embryology. In 1875 Ernst Haeckel observed the invagination processes in relation to the germ layer formation and proposed the gastrula as a stage of development (Beetschen, 2001; Haeckel, 1875) . This led to the work of Hans Spemann and Hilde Mangold that show the existence of an organizing centre during development (Beetschen, 2001; Spemann and Mangold, 1924) and the later work of Pieter Nieuwkoop (Beetschen, 2001; Nieuwkoop, 1969) that led to the concept of induction of a tissue by another. Eventually, this led to the understanding of the several steps of development after fertilization: morula, blastula, gastrula, neurula..... Later, the development new models such as the reaction-diffusion model of Alan Turing (Turing, 1952) or the positional information model of Lewis Wolpert (Wolpert, 1969) decipher the signals that shape and orchestrate development (Green and Sharpe, 2015).

1.2 Evo devo as a field of biology

Evo-devo takes its roots in this scientific revolution of the 19th century and subsequent discoveries, by filling the gap between development and evolution. Evo-devo is the comparison of developmental genes, gene networks, their regulation and the associated phenotypes to understand how their change can led to change in astonishing diversity of animal (or plant) that we observe today. In other word, "*explores the mechanistic relationships between the processes of individual development and phenotypic change during evolution*" (Müller, 2007)

1.2.1. Comparison of embryo in the 19th century

The idea of a link between evolution and development appeared early in the 19th century with the comparison of embryology of different species, mostly vertebrate species. Rapidly, naturalists as von Baer found out that early stages of development of well separated species exhibit some striking similarities and that specific trait of a given species form later in their development, it is the first von Bear law: "*the more general characters of a large group of animals appears earlier in their embryo than the more special characters*" (Goodwin et al., 1983)

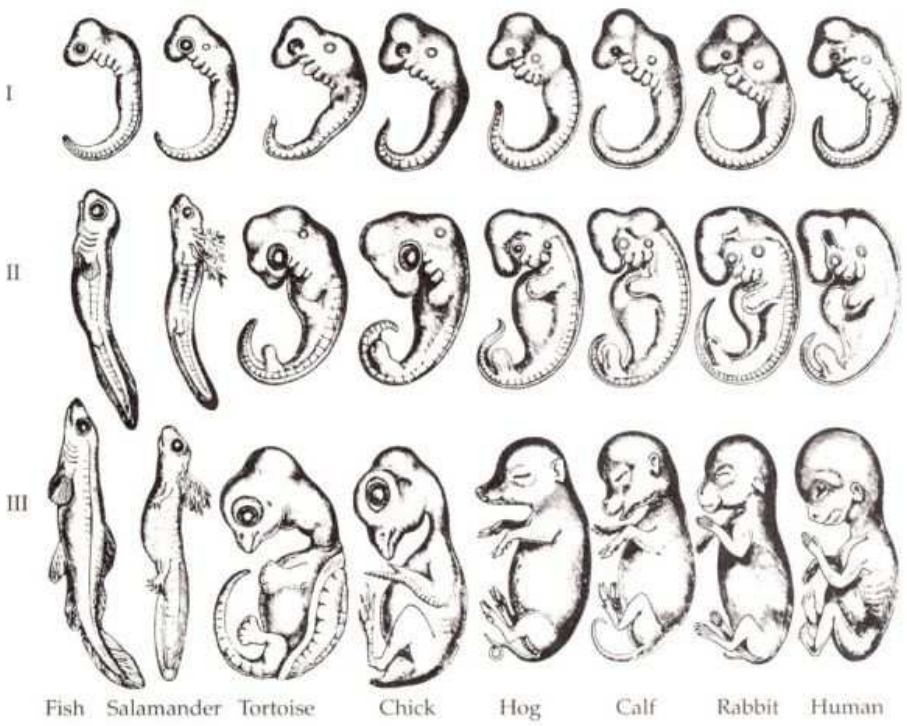

Figure 2: Reproduction of Haeckel's controversial developmental comparison of vertebrates. From left to right teleost fish, salamander, turtle, bird, pig, calf, rabbit and human. I: early somitogenesis, II: late somitogenesis, III: late embryogenesis. Adapted from Haeckel, 1866.

Haeckel performed an extensive work of comparative embryology (Figure 2) and popularized the idea that during its development, a species passes through the successive adult shape of its ancestor. It is his famous quote "*ontogenesis recapitulates phylogenesis*" (Haeckel, 1866) that popularize this idea. Nevertheless, other before him point out similarity between the fossil record and development (Agassiz, 1845; Goodwin et al., 1983). According to Haeckel, this is why a human embryo will first exhibit gill slits in the pharyngeal region, as in fish and later a tail, as in other mammals. Although Haeckel allowed the diffusion of Darwin's theory of evolution, his idea of recapitulation was a misconception and it keeps the idea of ladder-like and linear organisation of life. Von Bear already criticized this idea of recapitulation in 1829 and emphasized on the fact that "*Every embryo of a given animal form, instead of passing through the other forms, rather becomes separated from them*" (von Baer, 1828).

1.2.2. The same genes controlling the same structures

In the 1980's, the *hox* genes that controls the development of the fruit fly Drosophila melanogaster where discovered (McGinnis et al., 1984). Evo-devo developed as a field when, at the general surprise (Carroll, 2005), highly similar ortholog genes were identified in mouse (Duboule and Dollé,

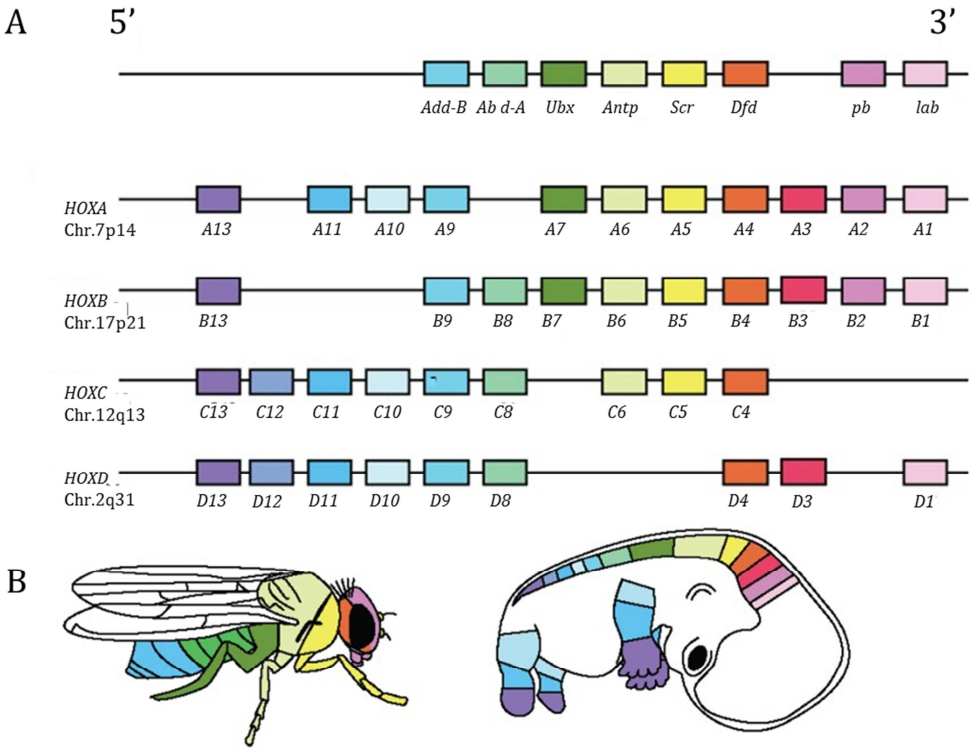

Figure 3: Hox genes in fruit fly and human. A organization of the Hox cluster in the fruit fly *Drosophila melanogaster* (upper panel) and the human *Homo sapiens* (four lower panels). B. Spatial expression of the Hox gene and associated developmental conntrol along the body axis of the fruit fly (left) and human (right). Adapted from Drumond et al, 2012.

1989; Graham et al., 1989) and were shown to have the same function. In other terms, genes in mouse and drosophila that originate from the same ancestral gene were similar enough to be recognized and display the same function during development. More surprisingly, not only the function, but also the organization of the *hox* genes is conserved between species. Strikingly, their position on the chromosome is reflecting the body part in which they are expressed (Figure 3; Durston et al., 2012; Graham et al., 1989) Other genes controlling the same structures in distant species were rapidly identified such as *eyeless* (in fly)/*pax6* (in vertebrates) that control the eye formation (Quiring et al., 1994 ; *dll* (in fly)/*dlx* (in vertebrates) that controls the growth of appendages (Panganiban et al., 1997) or *tinman* (in fly)/*nk2* genes (in vertebrates) that control heart formation (Bodmer and Venkatesh, 1998). These investigations became possible thanks to the ongoing progress of genetics and genomics that allowed a better understanding and manipulation of genes. Thus, it became clear that the same set of genes, the same toolkit, was controlling the same body part in distant animal, hence the paradox of evo devo: how, from essentially the same genes, in different species, is it possible to generate such diversity? This is the central question that evo devo is answering since the mid 1980's (Carroll, 2008).

1.2.3. Homology at the centre of evo devo

Homology is a core concept of evolution sciences and evo-devo, it proposes that "*characters found in different species are homologous if they are derived from the same character in their most recent common ancestor, regardless of similarity in form or function*." (Wagner, 2007). It is this definition that we will consider since it emphasizes the necessity of a historical continuum between characters to define homology. The textbook example often uses to explain homology is the forelimb of vertebrate (Figure 4; Wagner, 2007). This forelimb has several function like walking, grabbing, rowing or fling, it is different in shape and size and composed by a different number of bones. Nevertheless, those limbs are homologous since they can be trace back to the common tetrapod ancestor that has a forelimb (Schneider and Shubin, 2013) and the developmental processes controlling the limb

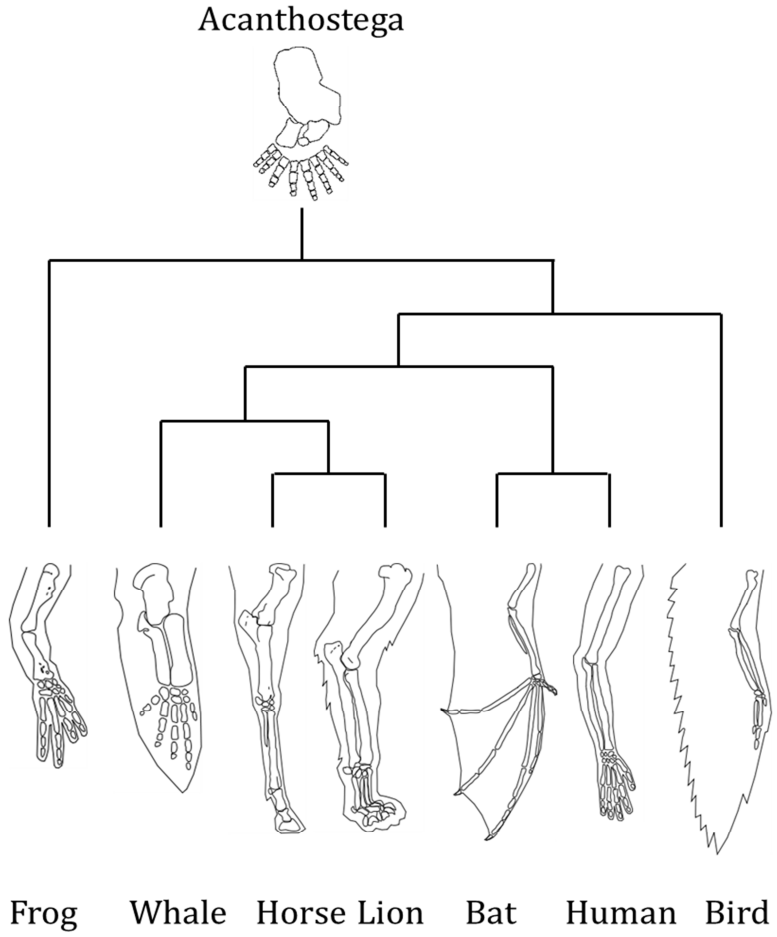

Figure 4: Vertebrate forelimbs illustrating the homology of this structure despite different functions. The forelimb of *Acanthostega*, tetrapods ancestor, is at the root for comparison. Adapted from Wagner, 2007; Schneider and Shubin, 2012.

development are shared between vertebrates (Hinchliffe, 2002; Zakany and Duboule, 2007). In contrast within these forelimbs, the wings of birds and bats, when considered alone, are not homologous because, despite a similarity in fonction (flying) they do not come as wings from a direct common ancestor. Scrutinizing their development and anatomy led to the conclusion that that they are the product of convergence, and not homology.

Therefore, it is not only the structure that can be defined as homologous, but also the gene pathways themselves (Wagner, 2007). For instance metabolic pathways are not involved in structure or shape formation but they are still inherited from ancestor, and therefore match our definition of homology. Thus if some core genes controlling any biological process can be proven as orthologs, therefore the process itself can be defined as homologous. This is has been done for the metamorphosis of chordate (Paris and Laudet, 2008).

2 Thyroid hormone Signalling

2.1. The thyroid hormone

Thyroid hormone is a major signaling hormone involved in many biological activities. Thus, it has been subject of many investigations. Mostly mammal based, with a medical perspective. Thyroid hormone was isolated in the beginning of the 20th century. In 1919, Edward Kendall described for the first time how he purified thyroxine, the so called active compound of thyroid, from pig thyroids (Kendall, 1919). This process was tedious since 33g of thyroxine was isolated from 3 tons of pig's thyroid. The first synthesis of the thyroxine was performed a few years later by Charles Harington and Gorge Barger (Harington and Barger, 1927). T3, another form of the hormone was isolated much later in the 1950's (Roche et al., 1953).

2.1.1. TH synthesis in the thyroid

The thyroid gland, is the organ which centrally synthetize TH in vertebrates. The thyroid exhibit a glomerular structure in which thyrocytes embed a extracellular colloid made of dissolved sugar in which the TH is synthesized (Figure 5). Thyrocytes are responsible for the uptake of anionic form of iodine (I^-) in the colloid as well as the synthesis of all the proteins necessary for the TH synthesis (van de Graaf et al., 2001). To understand how TH is synthetized, the simpler way is to follow the iodine.

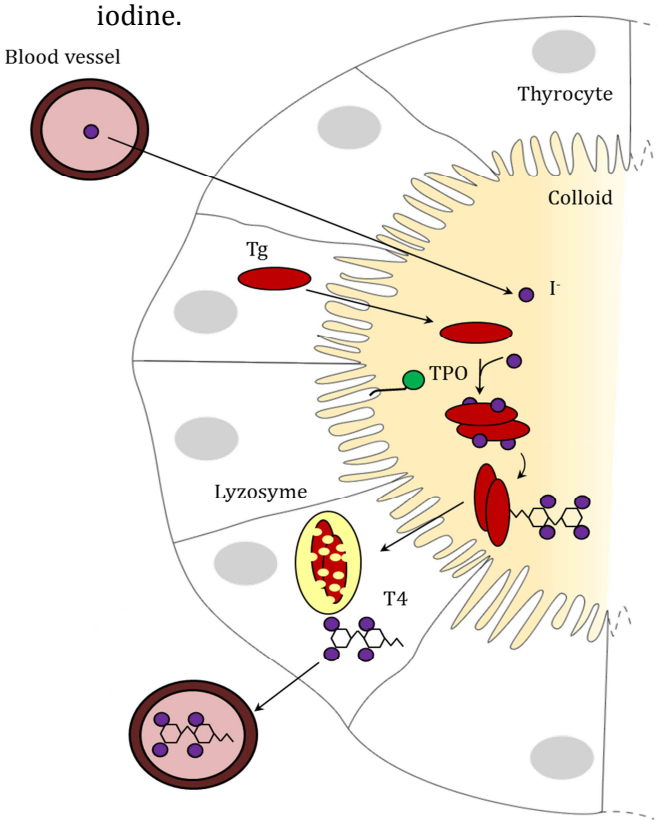

Figure 5: Synthesis of TH in the thyroid follicle. Iodine from the blood is up taken in the thyroid through SIS channel. The thyroglobuline (Tg) in synthetized by thyrocytes and exported in the colloid. Iodine is bound to Tg by the membrane-bound thyroid peroxidase (TPO), and T4 is formed still bound to Tg. Tg complexes re-enter the thyrocyte where they are degraded in lysosomes, T4 is synthetized during this process. T4 is then release in the blood stream. Adapted from van de Graaf et al, 2001.

Iodine comes from food, dissolves in the blood stream and is imported in the colloid by the thyrocytes through specific cell membrane channels (van de Graaf et al., 2001). Iodine is then coupled in the tyrosines of a very important protein, the thyroglobulin (Tg), by the membrane bound thyroid peroxidase (TPO).

Tg is a huge protein of about 2700 amino acids (Bergé-Lefranc et al., 1981), it harbour several domains called Tg repeat I, II and III. Tg I is repeated 11 times, Tg II 3 times and Tg II 5 times. These Tg repeats are extremely rich in cysteins indicating that Tg is a highly structured protein (Malthiéry and Lissitzky, 1987). Tg is also known to undergo a lot of post-translational modifications such as glycosilation that are necessary for its exportation to the colloid and its function (Kim and Arvan, 1998). Moreover Tg also harbours an Acetylcholine esterase like domain (ChEL-like) in its C-ter part. This domain is necessary for the export and the good conformation of the protein (Lee and Arvan, 2011; Lee et al., 2009). Although the Tg I repeat have been investigated (Novinec et al., 2006), little is known about the evolution of the whole protein. Tg are actively excreted by the thyrocytes in the colloid. During the process of TH synthesis, two Tg dimerize. Iodine atoms are coupled on some tyrosines of the dimer and two tyrosines are conjugated together by TPO (Gavaret et al., 1981; Magnusson et al., 1987) . This reaction allows the synthesis of two to four TH molecules per Tg dimer (Lee et al., 2009) . After the coupling, Tg dimers are imported by the thyrocyte, degraded in lysosomes and TH is released in the blood stream.

The resulting TH is a hormone made out of two benzene cycles harbouring iodine atoms, linked by an oxygen atom. The cycle carrying to remaining the amino acid group (NH2-CR-COOH) is called the inner ring and carries the iodine atoms on the carbons 3 and 5. The other ring is called the outer ring and carries the iodine atoms on the carbons 3’ and 5’ (Figure 6).

There are two similar molecules regrouped under the name of TH: T4 with 4 iodine atoms and T3 with 3 atoms. T4 is the more produced than T3 by the thyroid gland with an estimation of 80% of T4 for 20% of T3 (Nussey and Whitehead, 2013).

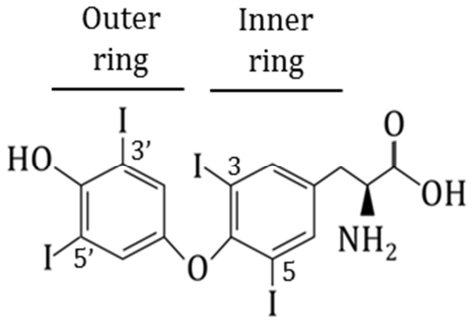

Figure 6: Thyroid hormone T4

2.1.2. The roles of TH

TH has a pleiotropic role that can be divided in three aspects in development, physiology and integration of environmental clues.

TH Metabolism is the most studied aspect of TH signalling. TH is involved in the control of the catabolism of fat and sugar and the resulting metabolic rate. Higher the TH levels induce higher energy expenditure and conversely (Mullur et al., 2014). TH is also associated with the thermogenesis in mammals (Lebon et al., 2001) and birds (Ikegami et al., 2015). Indeed, high TH level will tend to uncoupled the ATP synthesis chain in the mitochondria and thus increase the heat synthesis while increasing the global energy consumption (Lebon et al., 2001). Interestingly, this increase of metabolism is not restricted to mammals since some studies in zebrafish have shown that TH level are associated to energy expenditure in the muscles (Little et al., 2013). Interestingly, this

TH dependant metabolism is also linked to the food uptake. Hyperthyroidism is correlated with a slim phenotype despite an increased food uptake while hypothyroidism is associated with fat storage (Mullur et al., 2014).

TH is also involved in development processes and required for the maturation of several organs. As we explained before, it is acknowledged that TH controls chordate metamorphosis. Aside from this role in this critical development period, TH is also involved in less spectacular but nonetheless important processes. In human there is a peak of TH at birth (Erenberg et al., 1974), and TH is one of the first hormones dosed in the new-borns (Morvan-Dubois et al., 2013). Studies show that a defect of TH at birth or exposure to thyroid disrupting compounds can induce defect of development (Lopez-Espinosa et al., 2011) such as a syndrome of congenital and irreversible cretinism by impairing the brain development (Delange, 2001; Raiti and Newns, 1971). Many experiments have been performed in the mouse *Mus musculus* to understand the role of TH signalling during post-embryonic development (Flamant and Samarut, 2003). The framework established from these works shows that TH signalling becomes functional around birth and is critically important for the development of intestine (Flamant et al., 2002), bone (Göthe et al., 1999) and brain (Morte et al., 2002).

The last role of TH signalling that we will discuss here is the response to day lengthening. It is known that day length influences the physiology and the behaviour, particularly on the sexual level. In a lot of species ranging from salmon (Lorgen et al., 2015), to duck (Jacquet, 1997) or sheep (Bentley, 2008) the circulating level of TH rise as a consequence of this environmental changes.

2.1.3. TH derivatives

The metabolism of TH is important for the control of the signalling. As the thyroid synthetises mainly T4, the conversion of the T4 into T3, which has a higher biological activity, and the degradation of T3 in inactive forms is performed by specific enzymes, the deiodinases. Depending of what iodine atom is removed from the backbone, the deiodinases will therefore produce active compound or, at the opposite degrade the active hormone. As these enzymes are expressed in peripheral tissues and can respond to specific cues this ensures a fine tuned regulation of the T3 level in the tissue at each time and/or physiological situation.

2.1.3.1. Three deiodinases with different activities

There are 3 deiodinases in vertebrates, Dio1, Dio2 and Dio3 and each of them as different biological activities (Bianco and Kim, 2006; Köhrle, 1999). These enzymes are selenoproteins, meaning that one of their amino acid is selenocystein. This amino acid is structurally equivalent to a cystein in which the sulphur atom has been replaced by a selenium atom. This selenocystein is the active site of all three deiodinases and make a bond with the iodine atom that is to be removed from the TH.

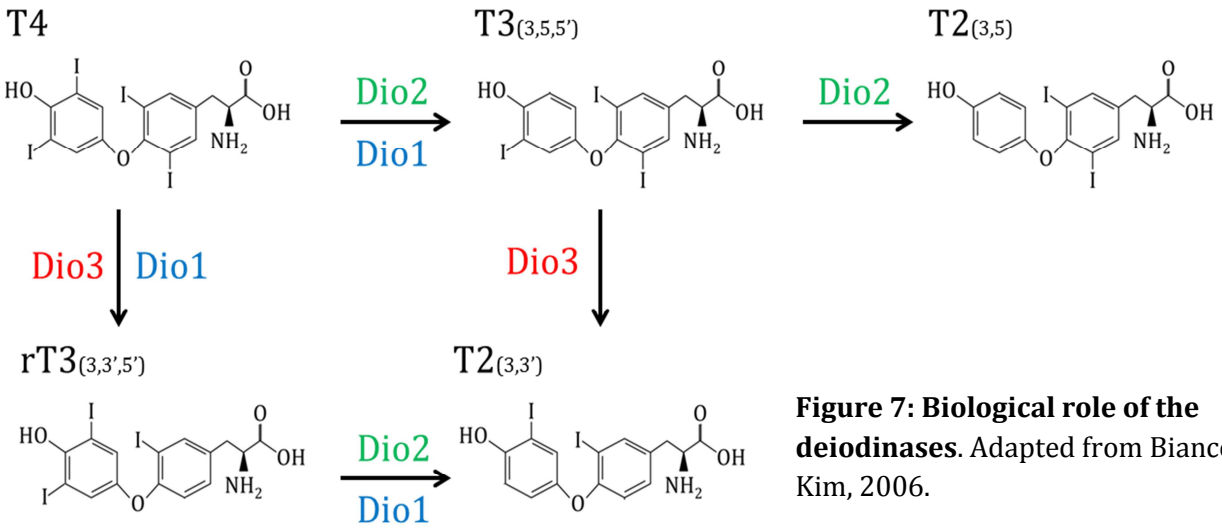

Figure 7: Biological role of the deiodinases. Adapted from Bianco and Kim, 2006.

The Dio2 (Figure 7), also called the outer ring deiodinase (ORD), catalyses the removing of iodine from the outer ring of the hormone. Therefore, it transform T4 into T3 (the 3',3,5-T3) and T3 into 3,5-T2 (Wassen et al., 2004). Its preferred substrate is T4. *Dio2* expression is strongly negatively regulated by T3 and induced by cold or overfeeding, that is to say physiological statuses that require an increase of TH signalling (Bianco and Kim, 2006). As T3 is the most biologically active form of TH and some recent evidence show that 3,5 T2 has a biological activity (Ball *et al.*, 1997; Mendoza *et al.*, 2013) . Dio2 is considered as an activating deiodinase.

Dio3 (Figure 7) has an inner ring deiodinase activity (IRD). Thus, it turns T4 into reverse T3 (rT3 or 3',5',3-T3) and T3 (3',3,5-T3) into 3',3-T2. Its preferred substrate is T3 over T4. (Wassen et al., 2004) *Dio3* expression is induced by T3 (Bianco and Kim, 2006) therefore ensuring that no excess of T3 is present inside tissues. Both rT3 and 3',3-T2 are considered as inactive. Thus, Dio3 is considered as an inactivating hormone.

The role of Dio1 (Figure 7), in comparison, is less clear. This enzyme exhibit both ORD and IRD activities but is thought to be less active than its more specialized counterparts. It preferred substrate is rT3 and its expression is positively regulated by T3 but also T2 (Baur et al., 1997) Kuiper et al., 2005). The biological role of this enzyme would ensure the background degradation of TH and the deiodination of its final compounds, as a scavenger enzyme.

Those three deiodinases with different roles allow, through an interplay of the expression of these enzymes, a precise regulation of the TH level in a given tissue (Bianco and Kim, 2006; Kuiper et al., 2005). Therefore, it is not the systemic level of TH that modulate the hormone action on a given organ or cell type, but the local action of Dio2 and Dio3 that allow the balance between the activation and the repression of the TH signalling (Köhrle, 1999). This regulation involves TH signalling itself since T3 represses the expression of activating deiodinase Dio2 and T3 enhance the expression deactivating deiodinase Dio3. This regulation by the deiodinase is supposed to be shared by all vertebrates since orthologs of deiodinases have been identified in their genomes.

2.1.3.2. Other derivative

There are other derivatives of TH that consist not only of variation in the number of atoms but also the amino acid group of the hormone. (Figure 8, Wu *et al.*, 2005). Here we will consider only two derivatives Tetrac and Triac. Tetrac (Tetraiodothyroacetic acid) is the result of the deamination of T4, thus it harbors 4 iodine atoms. Triac is the mainly produce by the deamination of T3, but can also be obtained by the deiodination of Tetrac (Wu & Visser, 1994).

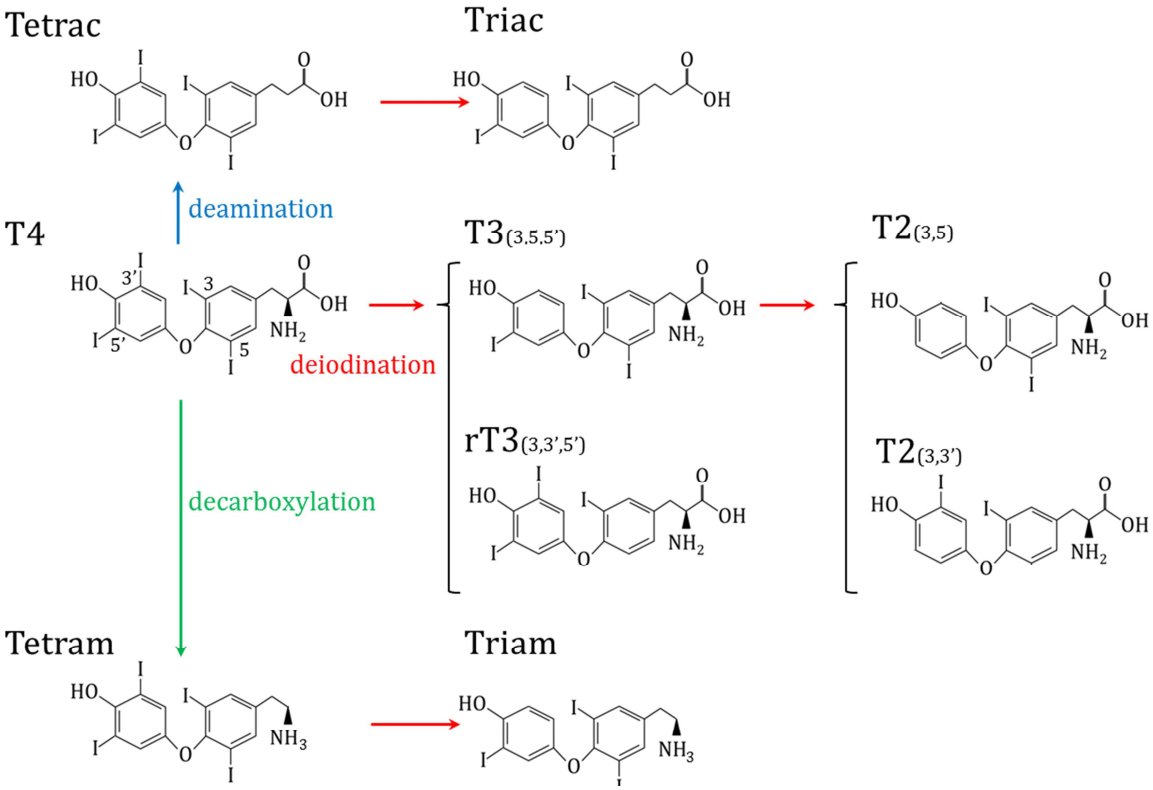

Figure 8: **TH derivatives**. Red arrows indicate deiodination, blue arrow deamination and green arrow decarboxylation from T4.

2.1.4. The neuroendocrine system

As explain earlier, TH is involved in metabolism, development and response to environment. It is the brain that integrates metabolic and environmental variations and induces the adjustment of the TH level in consequence (Fliers et al., 2014). The Hypothalamus-Pituitary-Thyroid (HPT) axis is the central regulator of TH signalling (Figure 9). The hypothalamus and the pituitary are involved in neuro-endocrinological system such as the Hypothalamus-Pituitary-Adrenal axis (HPA; Denver, 2009a) or the Hyphothalamus-Pitutary-Gonadal axis (Pierantoni et al., 2002). The centralization of those regulations allows the cross-talk, the feedback look and the interconnection of all those processes (Denver, 1997; Nakao et al., 2008). Thus, it is not a surprise that such an important hormone pathway as TH signalling is controlled by one of the oldest part of the vertebrate brain (Sower et al., 2009).

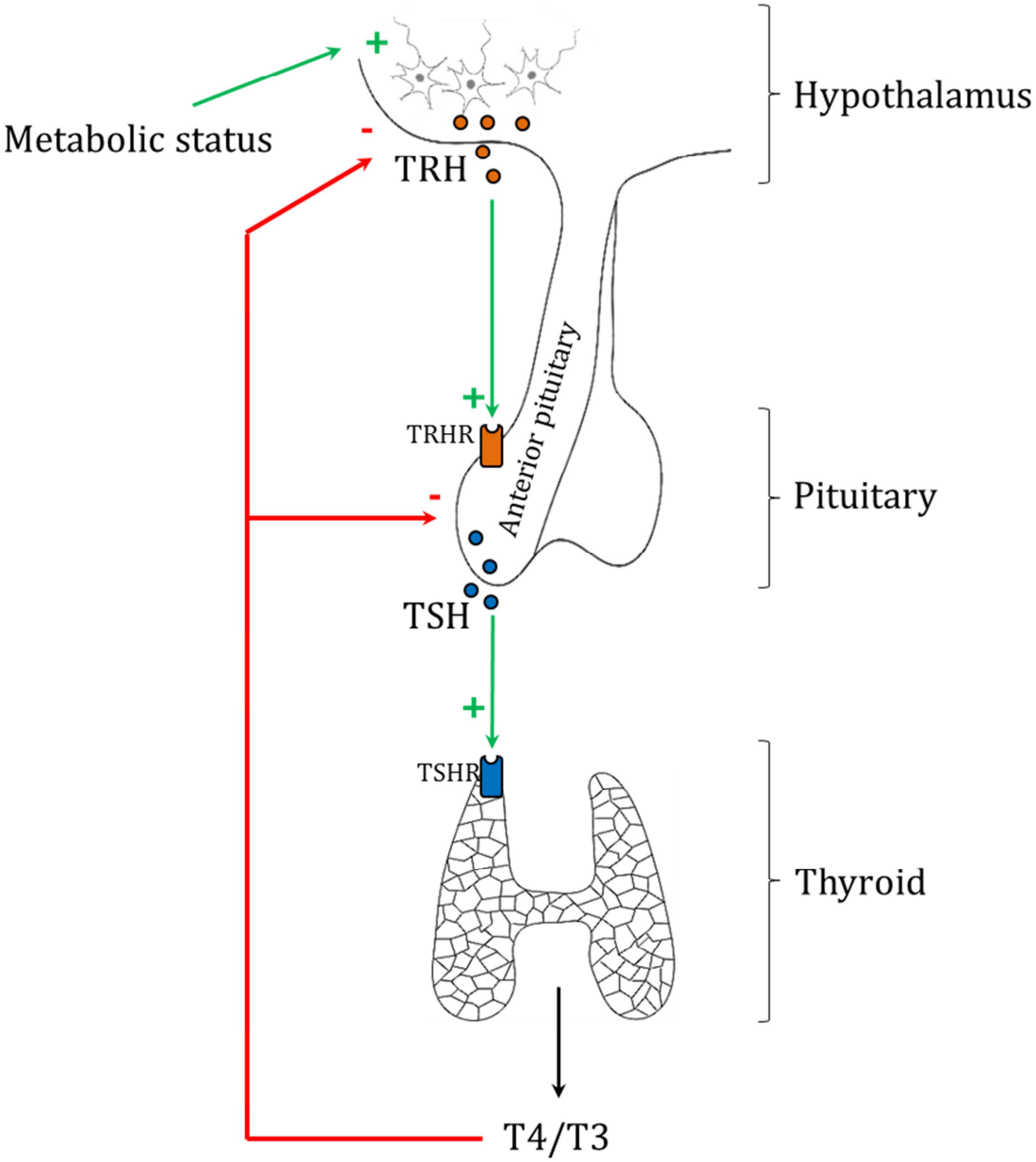

Figure 9: Regulation of the HPT axis. Environmental clues induce the release of TRH by the hypothalamus. TRH binds TRHR on the anterior pituitary which release TSH. TSH binds TSHR on the thyroid which release THs. THs have a repressive action on both the hypothalamus and the pituitary

2.1.4.1. The Hypothalamus Pituitary Thyroid axis

The hypothalamus is a neuroendocrine gland that sits just above the cerebral trunk. It secretes the Thyrotropin Releasing Homorne (TRH) on the blood stream that binds the TRH receptors (TRHR), particularly on the anterior pituitary. The pituitary is also a neuroendocrine gland that is located under the hypothalamus. When stimulated by the TRH, the anterior pituitary secretes the Thyroid Secretary Hormone (TSH), which binds on the TSH receptor (TSHR) of the thyroid gland. In response, the thyroid will release TH in the blood stream. The HPT axis is regulated by a negative feedback loop exerted by TH on the hypothalamus. High levels of TH inhibit the synthesis of TRH (Segerson et al., 1987) and TSH (Franklyn et al., 1988). In return, the stimulation of the thyroid is reduced. This reduces the global mount of circulating TH and ensure that the global level of the hormone never pass certain threshold (Figure 9).

2.1.4.2. The Hypothalamus Pituitary Adrenal axis

Environmental stress factors are integrated by the central nervous system. This signalling is transduce into hormonal secretion, namely the corticotrophin releasing factor (CRF) peptide. CRF is released by the hypothalamus, binds to the CRF receptor of the anterior pituitary. This gland releases the adrenocorticotropic hormone (ACTH) that triggers the release of glucocorticoids (GCs) by the adrenal glands. GC is the main effector of the acute stress response.

2.1.5. Outside of vertebrates

There is no organ thyroid outside vertebrates but there is a TH signalling since Triac has biological activity in the chordate amphioxus (Paris, Escriva, *et al.*, 2008). In this animal, six deiodinases have been identified (Klootwijk et al., 2011). Among those, one does not exhibit a selenocysteine but a cysteine at the active site. It is precisely this deiodinase that has a Triac specific activity. A functional homolog of vertebrate deiodinases has been identified in the tunicate *Halocynthia rorezti*. This homolog has a selenocysteine at the active site, as in vertebrate and has an outer ring deiodinase activity on T4 and rT3, as the vertebrate Dio2 (Sheperdley et al., 2004).

2.2. The thyroid hormone receptor

The thyroid hormone receptor (TR) belongs to the nuclear receptor superfamily (Figure 10). There are two paralogs in vertebrates TRα and TRβ. They are ligand dependant transcription factors that form heterodimers with their partner, RXR another nuclear receptor, to regulate the transcription of target genes.

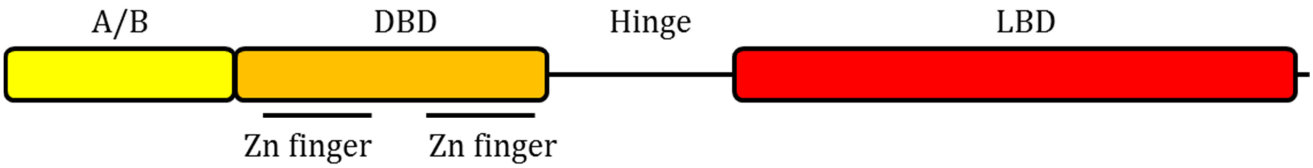

Figure: 10: Overview of the TR structure

2.2.1. Structure

2.2.1.1. The A/B domain

The A/B domain is an uncoiled region in the N-terminal part of the protein, it is about 40 amino acids long. This domain has a ligand independent transcriptional activation function, although the whole receptor is has a ligand dependant activity. The A/B domain is involved in protein-protein interaction, particularly with the co-activators it also give a cell specificity as this domain differs in the two TRβ isoform of mammals TRβ1 and TRβ2 (Aranda and Pascual, 2001). This domain is subject to phosphorylation that can alter the activity of the whole receptor (Tzagarakis-Foster and Privalsky, 1998). This domain is not well conserved across species.

2.2.1.2. The C domain: the DNA biding domain

Among all the TR domains, the DBD is the better conserved through evolution. The DBD is about 70 amino acids long and allow the binding of the receptor to the DNA thanks to two zing finger domains. A region of the DBD is involved in the heterodimer formation, with RXR. The DBD recognize several sequences as binding domain but its preferential biding sequence is AGGTCANNNAGGTCA, that is to say the nuclear receptors half-site AGGGTCA in direct repeat (same direction) separated by four random nucleotides. This site is called a DR4 for direct repeat 4 or a thyroid response element (TRE; Umesono et al., 1991). The DBD also binds on the TREpal, a response element with two half-sites in palindrome that was discovered first (Glass *et al.*, 1988). Within those zinc fingers, the P-box in the first zinc finger allows the recognition of the specific DNA half- site sequence by forming an α-helix that makes specific base contact in the major groove of the DNA molecule (Figure 11). A second important region in the DBD is interaction surface between the two monomers (TR and RXR) that specified the distance that the heterodimer recognize, here DR4 (Umesono and Evans, 1989).This interaction surface is determined by the D-box of the second zinc finger (Zechel et al., 1994). The ligand TR-RXR to DNA is ligand independent, meaning that TR always sits on DNA with or without ligand.

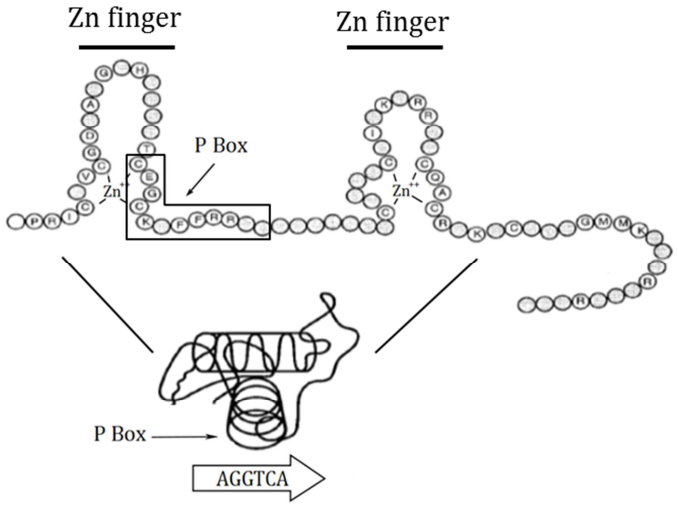

Figure 11: Detail of the DBD with the secondary and three dimensional structure of the DBD. The TR P-box makes contact with DNA on an half-site. Adapted from Aranda and Pascual 2001.

2.2.1.3. The D domain: the hinge

The hinge is an uncoiled portion of about 30 amino acids that allows the junction and the spatial articulation of the DBD with the LBD. It explains the pleiotropic binding of the TR-RXR heterodimer on direct repeats as well as on palindromic elements. It contains a nuclear localization signal that allows the translocation of the protein inside the nucleus (Aranda and Pascual, 2001).

2.2.1.4. The E domain: the Ligand Biding Domainv

The LBD is the larger domain of the TRs with about 270 amino acids. It is also the second most conserved domain after the DBD. The structure of the LBD contains a hydrophobic pocket in which the TH can dock. The unliganded form of the receptor is called the apo-TR and the liganded form the holo-TR. There are 12 α-helixes H1 to H12 that form the barrel-like spatial structure of the LBD (Wagner et al., 1995) with the helixes 3, 6 and 12 forming the biding pocket. When unbound, the helix H12 of the LBD is in an "open" conformation. When bound, some of the amino-acid in the inner face of the pocket will form some hydrogen bounds with the ligand which close the H12 helix and stabilized the whole holo-TR complex (Moras and Gronemeyer, 1998). This change of conformation triggers a major shift in the coregulatory proteins that interact with the TR/RXR. It allows the heterodimer to work as an on/off switch for the transcription of its target genes (Gronemeyer et al., 2004).

The apo-TR act as a transcriptional repressor and recruits co-repressors such as SMRT or NCOR that serve as scaffold protein for histone acetylases (Aranda and Pascual, 2001; Chen and Evans, 1995) . These acetylases induce the condensation of the chromatin in a closed state and therefore the repression of the target genes (Li et al., 2000). In contrast, holo-TR will recruits co-activators such as TRAP or p160 protein that will induce an open chromatin conformation and therefor the recruitment of the transcriptional machinery (Treuter et al., 1999). This allows the activation of target gene expression. This recruitment is possible as the LBD of the holo-TR makes a hydrophobic interface with the helixes H3, H5 and H12 that interacts with LXXLL motif of the co-activators (Oñate et al., 1995). This is a broad view of the transcription and the precise mechanisms are more complex.

The role of the repression of the apo-form is well illustrated by several studies showing that TR KO phenotypes are different from hypothyroidism phenotype (low or no TH level). This data show clearly that the absence of TH induce much worse effect than the absence of the receptor. (Flamant and Samarut, 2003; Flamant et al., 2002). Indeed when there is no ligand the apo-receptor is able to repress transcription and this may have detrimental effects whereas without the receptor there is a milder phenotype resulting from the absence of TH regulation of target genes. This ligand dependant regulation of the transcriptional activity is known as the canonical pathway (Figure 12).

2.2.1.5. The F domain

The last domain of the TR is the F domain at the very C-terminal portion of the protein. This domain is absent in TR. Nevertheless, in one of the TR isoform called TRα2, there is a long F-domain that disrupts the H12 helix. Thus the binding pocket cannot properly close in the presence of TH, making this isoform a constitutive repressor of gene expression (Katz and Lazar, 1993). This illustrates the importance of alternating spicing in TR activity.

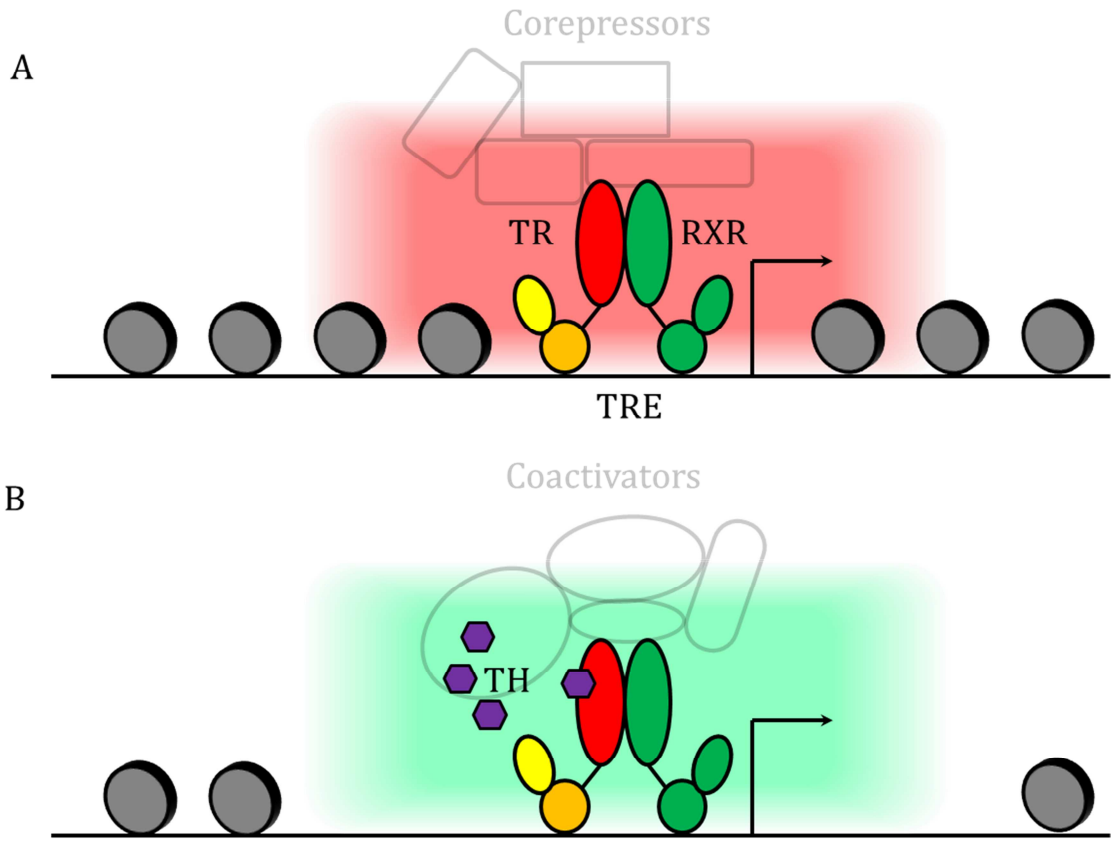

Figure 12: Principle of TR signaling. A. Repressive state: when no hormone is bound, the TR in partnership with RXR recruits corepressors that close the chromatin and repress target genes. **B.** Active state: when the hormone is bound, the TR in partnership with RXR recruits coactivators that open the chromatin and enhance the expression of target genes.

2.2.2. TR duplication and consequences

There are two paralogs of TR in vertebrates encoded in two different locus, *TRα* and *TRβ*., that allow a differential regulation of the TRs. These paralogs originate from the two rounds of whole genome duplication that happened at the basis of all vertebrates (Kuraku et al., 2009; Smith et al., 2013). The duplication allows the divergence of the two genes copies in terms of amino acid sequence and regulatory sequence. The two phenomena happen with the TRs. *TRα* and *TRβ* regulate different subset of genes, when tested in the same chromatin context, indicating that they have different intrinsic properties (Chatonnet et al., 2013). On the othjer hand *TRα* is expressed in the early development whereas *TRβ* is expressed later (Forrest et al., 1991) indicating difference of regulation. As a result the TRs regulate different physiological function.

In teleosts, there are two *TRα* genes *TRα-A* and *TRα-B* originating from a teleost specific whole genome duplication (Bertrand et al., 2004; Marchand et al., 2001) Those genes are also differentially expressed as exemplified in the flounder *Paralichthys olivaceus*, where *TRα-A* is express earlier at a higher level than *TRα-B* and *TRβ* (Yamano and Miwa, 1998).

2.2.3. TR affinity for TH derivatives

All the TH derivatives do not have the same biological activities. This can be explained by the availability of the derivative to enter the cell orthe affinity for the receptor or the half-life of the derivative. T3 has a 10-fold higher affinity than T4 for the receptor (Chopra, 1996). Moreover, it is taken up in cells at a faster rate than T4 (Everts et al., 1996), thus the affinity to the receptor and the bio-availability of T3 is superior to T4. Tetrac is has a very low biological activity with a short half-life, and is not considered a biologically relevant (Pittman et al., 1980). Triac, has an higher affinity for the TR than T3 (Wu et al., 2005, 2008) . Nevertheless, it is found in very low amount in the plasma. It has a very short half-life. Overall it has a lesser biological activity than T3 (Goslings et al., 1976)

2.2.4 Inverse regulation

All the genes do not respond to TH signalling according to the model developed earlier. Some genes are negatively regulated by TR in presence of TH and positively regulated in absence of the hormone. It is the case for the genes *TRH* and *TSHβ* (Sasaki et al., 1999; Satoh et al., 1999) that are involved in the neuroendocrine control of TH level. For these genes, the Holo-TR recruits a deacetylase complex that close the chromatin conformation in presence of TH. Unexpectedly studies have shown that both functional DBD and LBD are required for both positive and negative regulation of TR (Ortiga-Carvalho et al., 2005; Shibusawa et al., 2003). The negative regulation by TR is still an open question. The binding of a TR monomer on a half-site TRE that recruit specific co factors might be an explanation (Satoh et al., 1999). An alternative hypothesis is a differential DNA

2.2.5. The non-genomic pathway

Accumulating evidence reveal the existence of a non-canonical pathway called the non-genomic pathway. In this case, receptors are located on in the cytoplasm and not in the nucleus. Three independent non-canonical pathways have been described, although they are not well understood yet (Davis et al., 2008) . T3 can bind TRβ in the cytoplasm which activates with PI3K and induces a fast phosphorylation response in vascular endothelial cells (Martin et al., 2014; Hiroi et al., 2006). T4 can induce actine polymerisation through the ding of the isoform TRΔα1 (mammals specific isoform of TRα truncated in the N-ter part, unable to bind DNA) in astrocytes (Siegrist-Kaiser et al., 1990). Intriguingly, T4 and T3 can also bind directly the integrin αvβ3 which induces the MAPK pathway in glioma cells (Davis et al., 2006). Overall, these actions seem to be cell specific, still unclear and sometimes controversial. Non-genomic effects appear marginal in comparison of the canonical pathway.

3. Metamorphosis in chordates - state of the art

Metamorphosis is a post-embryonic life transition event that is observed in many animal species. The name comes from the greek meta- (*μετά*) meaning true, after or beyond and morphos (*μορφή*) meaning from or shape and originally describes a developmental step during which an individual undergo a dramatic reshaping. Metamorphosing has been fascinating scientists but also philosophers and poets for a long time. In many old texts as the Epic of Gilgameh (George, 2010), the Ovid's Metamorphoses (Ovide, 2005) or the werewolf folklore, some characters, humans, gods or others undergo a metamorphosis and transform into something else. All this imagination might took its roots into the observation of real examples of metamorphosis like the transformation of a tadpole into a frog, or a caterpillar into a butterfly. Eventually, the fascinating metamorphosis was tackled by science.

The scientific definition of metamorphosis has not reached a consensus and is still debated (Bishop et al., 2006). For our purpose, we will consider the metamorphosis as **the transition period between a larvae and a juvenile or adult where the individual undergo spectacular morphological, physiological, behavioural and ecological modifications** (Laudet, 2011). Thus, it is a post-embryonic developmental process. For our purpose we define the larva as the animal resulting of the embryogenesis with some degrees of autonomy. The juvenile is defined as an adult-like individual except for the sexual function and therefore unable to reproduce. It must be stressed however that in some cases (*e.g.* insects) the juvenile is immediately sexually mature. As we are discussing biological processes, there are of course exceptions or peculiar traits in the development of some species that blur this definition. This will be discussed further. In addition, the question of the definition of a metamorphosis outside metazoan, such as plant (Bishop et al., 2006), is beyond the scope of this thesis.

Metamorphosis is described in many taxa (Nielsen, 2000) such as annelids, molluscs, arthropods, sea urchin, sea squirt, fishes, amphibians,… Most of our knowledge, from which we build our definition of metamorphosis, comes from the study of two of these taxa: the amphibians and the insects. We will focus and develop the case of the amphibian and consider the insects later in the discussion.

3.1. Amphibians: the model of vertebrate metamorphosis

Amphibians, particularly the anurans, are the best known models of vertebrate metamorphosis with more than 6000 scientific articles discussing and investigating this process. In most of the cases, it involved the transition of a tadpole, a water living, water breathing herbivorous larva into a frog, a land living and air breathing carnivorous juvenile.

3.1.1. Historical perspective

3.1.1.1. Gudernasch 1912

The historical experiment that gave the first hint about the physiological mechanisms that underlie amphibian metamorphosis was performed by Joseph Gudernatsch in 1912 in its "*Feeding experiment on tadpoles*" publication. As the name of the publication suggests, he fed tadpoles of the frogs *Rana temporaria* and *Rana esculenta* with different horse body parts such as liver, muscle, ovary, testis and hypothalamus. and observed the resulting effects on tadpole development. He found that feeding

tadpoles with horse thyroid induces a precocious metamorphosis. The tadpoles stop growing to undergo metamorphosis and it was noticeable in that "*every change in the body form set in almost simultaneously in all the animals*" (Gudernatsch, 1912). If it was already known at that time that thyroid gland was a secretory organ, this work is the first demonstration that the secretion of a gland can control amphibian metamorphosis.

.3.1.1.2. Allen 1918

Subsequently, in 1918, Bennet Allen performed the first thyroidectomy of *Rana pipiens* tadpoles (Allen, 1918). This prevented the metamorphosis of these tadpoles into juveniles. However, feeding these tadpoles with thyroid extract allow the tadpole to undergo metamorphosis and rescues their normal development. This work shown that thyroid was necessary for the metamorphosis of amphibians.

3.1.1.3. Tata 1966

In 1966 Jamshed Rustom Tata showed that isolated tail of *Rana temporaria* grown in culture imitates a metamorphosis-like response when treated with TH: they regressed (Tata, 1966). Moreover, when co-treated with drugs inhibiting RNA or protein synthesis, this regression was inhibited. Therefore, he shows that the TH induces regression during metamorphosis was not only the result of the passive death of tail cells, but an active process under gene control that need RNA transcription and protein translation.

3.1.1.4. Leloup and Buscaglia 1977

In 1977, that Jacques Leloup and Marino Buscaglia monitored for the first time the level of TH during the metamorphosis of *Xenopus laevis* (Leloup and Buscaglia, 1977). This paper show that TH level begin to rise at the onset of metamorphosis and peak at the climax of metamorphosis, when the tadpoles exhibit the larvae (*e.g*. tail) and juveniles (*e.g.* limbs) features at the same time. It was the first description of the physiological change happening during amphibian metamorphosis.

3.1.1.5. The role of the TRs

After the cloning of TRα and TRβ of *Xenopus laevis* (Yaoita et al., 1990), several teams studied the level of expression of those genes at local and systemic level during *Xenopus* metamorphosis (Eliceiri and Brown, 1994; Kawahara et al., 1991; Yaoita and Brown, 1990). Later, transgenic experiments with dominant negative TRs show that the transformations induced by TH during the amphibian metamorphosis were mediated by the TRs, and that the TRs were compulsory for the metamorphosis (Buchholz et al., 2003; Schreiber et al., 2001). *TRβ* expression is concomitant with the TH level described by Leloup and Buscaglia (Leloup and Buscaglia, 1977), rising at the climax of metamorphosis. A TRE is found in the promoter of *TRβ* but not *TRα,* allowing its positive self-regulation that plays a critical role in amphibian metamorphosis (Machuca et al., 1995) and explain how the *TRβ* expression follows the TH level. However, *TRα* expression is different. This gene is

highly expressed during the early development of the tadpole, long before the onset of metamorphosis.

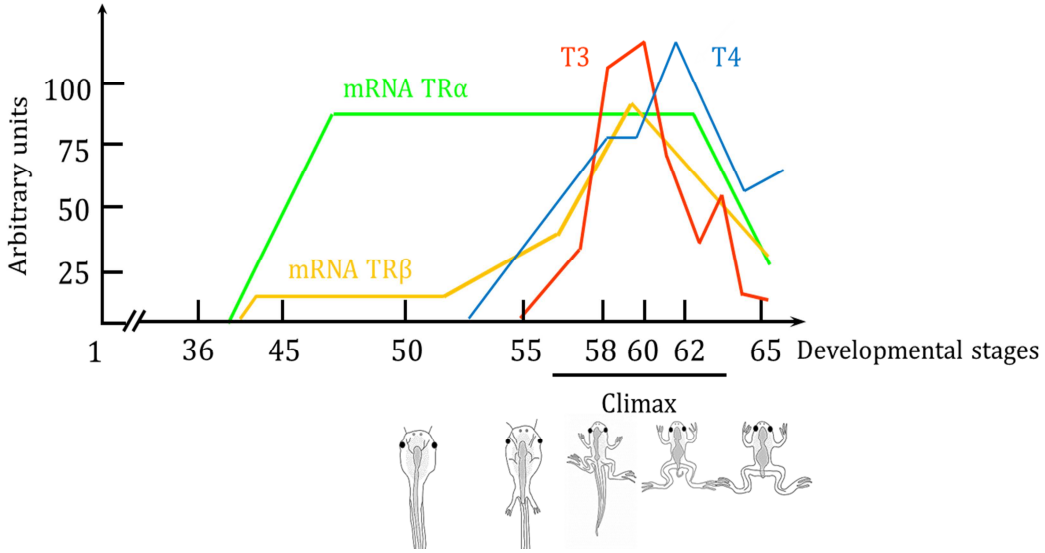

Figure 13: **Expression of *TRα* and *TRβ* and level of T4 and T3 during the developpement of *Xenopus tropicalis***. Arbitrary units are on the x-axis and developmental time on the y axis given in Nieuwkoop-Faber stages. Adapted from Buscaglia and Leloup 1987; Eliceiri and Brown, 1994.

3.1.2. Metamorphosis is a complex event

Altogether, this corpus of studies, with many others (Brown and Cai, 2007; Tata, 2006), allows us to establish what happens during amphibian development and leads to their metamorphosis (Figure 13). After organogenesis and prior *Xenopus* metamorphosis, *TRα* expression increases while there is no detectable TH in the tadpole. This results in the receptor repressing its target genes, which prevents the animal from undergoing a precocious metamorphosis. *TRβ* also has the same role in a lesser extent (Havis et al., 2006). When the metamorphosis is precociously provokes, or when it naturally occurs, *TRβ* expression start to rise with TH level, as it is self-induced, by the hormone (Yaoita and Brown, 1990). This positive loop coordinates the peaks of TH synthesis and *TRs* expression that reach their maximum at the climax of the metamorphosis. The mechanism breaking this positive loop, and decreasing both *TRs* expression and TH level, however is less understood. It may lies in the establishment of the negative feedback loop of TH on its own synthesis by the thyroid. Intriguingly, the model of unliganded TRs repressing the metamorphosis in early development is discussed (Morvan-Dubois et al., 2013). Some evidences show that TRs signalling is functional in early embryo, prior metamorphosis and has a developmental role (Fini et al., 2012) .Thus there is an apparent contradiction, which is not solved yet, highlighted the fact that TH signalling is much more complicated than anticipated.

3.1.2.1. Proliferation and apoptosis at the same time

Many organs and tissues of an amphibian undergo an extensive remodelling, under TH control during metamorphosis (Brown and Cai, 2007). Some structures undergo apoptosis whereas other are protected (Coen et al., 2001). Intestine changes from an herbivorous long coiled and villosity free intestine toward a carnivorous short, uncoiled and structurally complex one (Figure 14; Schreiber et al., 2005; Su et al., 1997). The skin changes to handle aquatic then aerial environment (Schreiber and

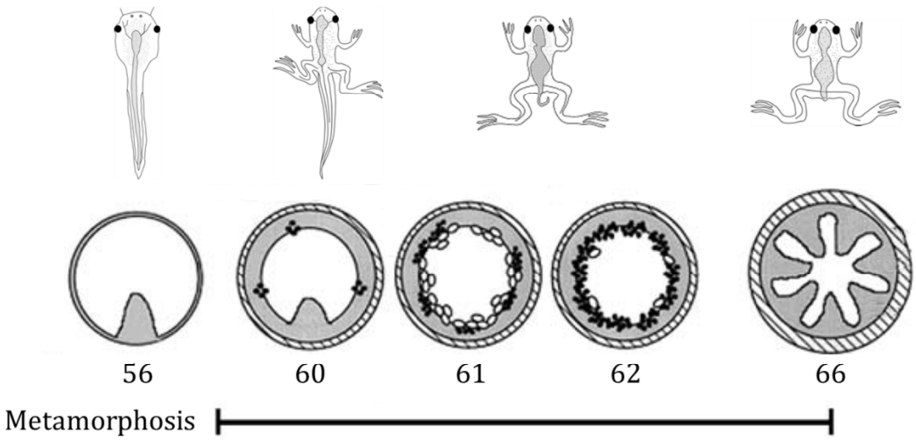

Figure 14: *Xenopus* intestine during metamorphosis. The developmental sateg in Nieuwkoop-Faber stages is given on the Y-axis. Adapted from Hasebe et al. 2013

Brown, 2003). The gills and the tail regress and disappear (Brown et al., 1996) . The inexistent limbs develop, with all the bones, muscle and neuronal remodelling it underlies (Brown et al., 2005). Tadpole erythrocytes, produced in the liver, undergo apoptosis and adult erythrocytes, produced in the bone marrow, proliferate (Nishikawa and Hayashi, 1999). Internal organs such as the eye or the digestive track are extensively remodelled (Mann and Holt, 2001; Schreiber et al., 2009). This is a fascinating situation since a peak of a single hormone is apparently controlling extremely different organ responses such as cell proliferation, cell differentiation or cell death. How is this achieved?

Each tissue expresses its own set of genes, has its own chromatin configuration and therefore will response in a specific way to TH signalling (Kulkrani, 2013). Furthermore, *TRα* and *TRβ* are differentially expressed and do not control the same of target genes in a given tissue (Chatonnet et al., 2013). The local expressions of the deiodinase have a critical role in the fine-tuned control of metamorphosis and this is well exemplified by the tail regression and limb growth (Becker et al., 1997; Kawahara et al., 1999). First, *dio2* is expressed in the limb buds, which locally turns T4 into T3, and so enhance the TH signalling. As a result, the limbs grow. At the same time, in the tail, *dio3* is highly expressed. The enzyme turns T4 into rT3 or T3 into 3',3-T2 which are inactive derivative of TH (Becker et al., 1997) . Thus, there is no or few TH signalling in the tail, which is preserved. As a consequence, limb and tail coexist. Later in metamorphosis, *dio3* expression changes in the tail in favour of *dio2*, the local level of T3 rises which induces the apoptosis of the tail cells and the regression of the organ at the climax of metamorphosis (Cai and Brown, 2004). The local control of TH by the deiodinases explains how a systemic signalling can have opposite effect on different organs. This regulation of the local level of TH also happens in fishes as the *dio2* and *dio3* are also differentially expressed during metamorphosis (Campinho et al., 2012; Isorna et al., 2009).

3.1.2.2 Environmental control of metamorphosis and stress

In tadpoles, environmental condition can trigger metamorphosis (Denver, 2009). Experiments consisting in lowering the water level in which tadpoles are developing resulted in precocious metamorphosis of these tadpoles. (Denver, 1997; Semlitsch and Caldwell, 1982). Similarly, presenting predator cues such as smell, or actual predators, trigger a precocious metamorphosis. This illustrates the tight coupling between the HPT and HPA axis, working specifically during metamorphosis. Indeed contrary to mammals or birds, it has been shown that, in amphibian tadpoles the CRF is a potent TSH inducer (Denver, 1988; Malagón et al., 1991). Thus, in stressed tadpole the activation of CRF stimulates both the release of both ACTH and TSH which in turn stimulates the secretion of TH and triggers metamorphosis. Concurrently, ACTH controls the release of the GCs (Figure 15). Interestingly, GCs, which binds the nuclear receptor, are required for amphibian metamorphosis (Kikuyama et al., 1982). This illustrated the subtle cross-talk between these two pathways.

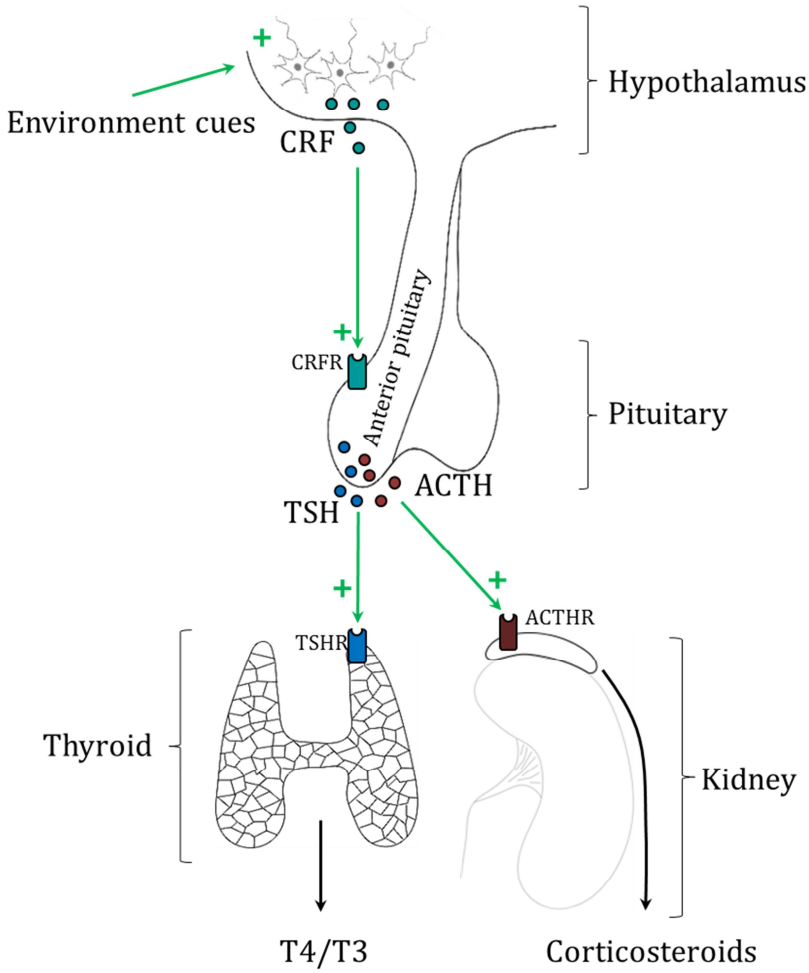

Figure 15: Regulation of the HPT axis in the amphibian tadpole. Environmental clues induce the release of CRF by the hypothalamus. CRF binds CRFR on the anterior pituitary which release both TSH and ACTH. TSH binds TSHR on the thyroid which release THs. ACTH bonds ACTHR on the surrenal glands that release corticosteroids. Both of these signallings are at play during amphibian metamorphosis. Adapted from Denver, 2009.

Nevertheless, some aspect of this regulation remains uncertain. For instance, how does the adult HPT axis set up after metamorphosis? TRH fails to induce TSH secretion in tadpole (Groef et al., 2006), whereas, it work well in adults (Castaño et al., 1992). Furthermore, how the TH level can rise so high during tadpole metamorphosis? We can understand that TH feedback cannot regulate the CRF level. (in contrast to TRH). But it has been shown that the negative feedback of TH on TSH works at the very beginning of tadpole metamorphosis (Manzon and Denver, 2004). These are still open questions that remain to be solved.

3.1.2.3. Intrinsic factors

If stress can trigger metamorphosis, some internal factors are also compulsory for the achievement of amphibian metamorphosis. At the beginning of metamorphosis, tadpoles feed and gain weight (Gramapurohit et al., 1998; Kuzmin, 1997). However, at the climax, tadpoles drastically reduce their food intake, and sometime stop it, because of the extensive remodelling of the feeding apparatus and digestive tract (Kuzmin, 1997). Thus, metamorphosis is an energetically demanding process and fat accumulation just prior the climax is required for metamorphosis (Wright et al., 2011). Interestingly there is a cross-talk between extrinsic and intrinsic factors on the influence of metamorphosis since food deprivation increases the GCs level, and in a lesser extent the CRF levels in tadpole but not in juveniles (Crespi and Denver, 2005). Moreover, CRF injections reduce the food intake in amphibian tadpole but GCs increase it, indicating a complex regulation system between stress, food and development (Crespi and Denver, 2004).

Early treatments of high dose of TH in *Xenopus laevis* show that tadpole can undergo metamorphosis really early in their development (stage NF46/NF47, around 1 weeks old), weeks before the natural occurring metamorphosis (stage NF56, around 6 weeks old; Brown and Cai, 2007; Tata, 1968). Evidence based on iodine accumulation suggests that thyroid accumulate iodine and become functional around one week old (Brown and Cai, 2007; Opitz and Kloas, 2010; Tata, 1968). Therefore there is latency between the gland becoming functional and the onset of metamorphosis (Brown and Cai, 2007) Thus, the growing and the maturation of the gland itself constrain the onset of metamorphosis.

3.2. Metamorphosis in teleost

If amphibians are the most studied taxon for metamorphosis, other vertebrates also undergo metamorphosis and many examples come from the teleosts. Teleosts is the most diverse taxon among vertebrates with about 30 000 species estimated (Near et al., 2012) and they undergo metamorphosis during their development.

3.2.1. Metamorphosis in flatfish

Flatfishes (Pleuronectiformes) are subjected to a spectacular metamorphosis during their development. The larvae of flatfish are pelagic, living in the water column and exhibit left-right symmetry. The adults, however, are benthic animal with a strong left-right asymmetry, one of their sides facing the water column and the other the ground (Figure 16; Einarsdóttir et al., 2005; Isorna et

al., 2009; Schreiber, 2006). This change of symmetry occurs during the metamorphosis where one of the eyes migrates on the side that will face the water column. During this event, the fish is still able to swim and, in some extent, feed despite the huge craniofacial remodelling occurring. This event is under the control of TH as the treatment with TH induces a precocious metamorphosis and the treatment with goitrogen, chemical compounds inhibiting the TH synthesis, delays the metamorphosis (Schreiber, 2006) In the Atlantic halibut *Hippoglossus hippoglossus*, TH levels, as the expression of the *TRs* peaks at the climax of the metamorphosis (Galay-Burgos et al., 2008). *Dio2*, the activating deiodinase, is highly expressed just before the onset of metamorphosis and *Dio3*, de deactivating one, is more expressed at the end of metamorphosis (Campinho et al., 2015). Thyroid gland activity reaches its maxima at the climax of the metamorphosis while the neuroendocrine cells within the pituitary gland, that centrally controls TH signalling proliferate (Einarsdóttir et al., 2005). In other flatfish, the same phenomenon of the switch of expression of deiodinase has been observed as in the Senegalese sole *Solea senegalensis* (Isorna et al., 2009).

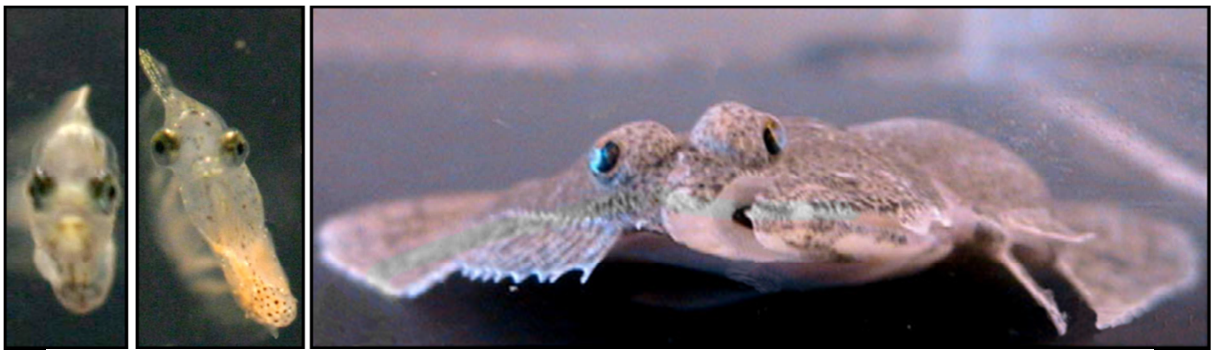

Figure 16: **Remodelling of the Athlantic halibut *Hippoglossus Hippoglossus* during its metamorphosis.** From left to right, symmetrical larva, larva during metamorphosis and assymetrical adult. Adapted from Schreiber, 2006.

The role of the neuroendocrine system in fish metamorphosis is reminiscent to the amphibian situation. Some evidences show that, CRF (Larsen et al., 1998) and TRH (Chatterjee et al., 2001) stimulates TSH and TH release similarly to amphibian. Furthermore, a study in the Senegalese sole *Solea senegalensis*, show that TH exerts no feedback on CRF nor TRH but a direct feedback on TSH (and other TH related genes such as Tg; Campinho et al., 2015). Nevertheless, the details of the HPT regulation in fishes are not well understood.

3.2.2. TH metamorphosis in teleosts: mainly aquaculture models

The role of thyroid hormone in fish development has been over the year studied in many fishes. The Pacific Bluefin tuna *Thunnus orientalis*, (Kawakami et al., 2008), the Japanese eel *Anguilla japonica* (Yamano et al., 2007), the Japanese flounder *Paralichthys olivaceus* (Yamano and Miwa, 1998) and the Orange-spotted grouper *Epinephelus coioides* (de Jesus et al., 1998) are among the best studied teleost species for which a TH peak correlates with the metamorphosis. In the zebrafish *Danio rerio*, the metamorphosis is very discreet and only a few publications investigate the endocrinology of this event (Brown, 1997; Liu et al., 2000; McMenamin et al., 2014). However, it is only in a few fish species, mainly flatfishes, that the levels of the hormone as well as the receptors were investigated (McMenamin and Parichy, 2013). The role of TH in the metamorphosis of teleosts is highly

reminiscent of the metamorphosis of amphibians, but the molecular details are much less known. Given that metamorphosis is a key step in the development and growth, it is not surprising that most studies focus on species that are currently developed in aquaculture. Thus, aside from those species, metamorphosis in teleosts is poorly characterized in regard of the diversity of this taxon. In particular the role of the environment in the triggering of the metamorphosis is poorly known.

3.3. Variations on the theme of metamorphosis

With more than 60 000 vertebrate species (Hoffmann et al., 2010), evolving since the Cambrian explosion, more than 500 million years ago (Budd, 2013), it is not surprising that, among species undergoing metamorphosis, there are a lot of variations. We will consider here the heterochrony of metamorphosis, that is to say, the change of timing or sequential order of the metamorphosis in respect to the other developmental events and the intensity of the phenomena.

3.3.1. Neotenia and Paedomorphism

Paedomorphism is an evo-devo concept which describes that adults of a given species retain characters of the larva of their ancestor. This has been well studied in apes in which the hairless body and face of the human *Homo sapiens*, as well as other feature as the skull shape is generally considered the retention of juvenile characters from the common ancestor of all apes, in regards of the juvenile and adult stage of its closest relatives (Leigh and Shea, 1996). Peadomorphism can be achieved by two ways. In neoteny, the somatic development is slowed down when compared the ancestral species meaning that juvenile traits remain at the adult age of a given species. In progenesis, the sexual maturity is accelerated and occurs before the somatic development of adult traits of the ancestral species. In amphibian, two examples of heterochrony involving neoteny and metamorphosis have been studied (Laudet, 2011).

3.3.1.1. The facultative metamorphosis of the axolotl.

The axolotl *Ambystoma mexicanum* is not only the cutest as well as weirdest example of what a salamander can be, but is also a wonderful example of neoteny. Salamanders belong to the urodela. In this taxon, the larvae exhibit external gills and tail fin while the adult have lungs and lose their tail fin. Contrary to other salamanders, adult axolotl retain their juvenile features: external gills, tail fin and larval pigmentation (Brown, 1997). Nevertheless, after puberty, they are true adults since they can reproduce. Interestingly, exogenous treatment with TH will induce the metamorphosis of the axolotl: the gills and tail fin shrink and the adult pigmentation develop (Brown, 1997). The TRs of the axolotl are fully functional (Safi et al., 2003) and TH treatment induces transcriptomal response similar to what is observed during the metamorphosis of other amphibians (Page et al., 2009). As metamorphosis can be artificially induced, the axolotl is considered as a facultative neotenic animal.

3.3.1.2. The obligatory neotenic Mudpuppy

The mudpuppy *Necturus necuturus* presents an obligatory neoteny. This salamander is able to reproduce while retaining larva feature, as for the axolotl, but it is impossible to induce its metamorphosis with TH treatment. However, the TRs are expressed, functional and control the expression of classical target genes that respond during amphibian metamorphosis (Safi et al., 2006; Vlaeminck-Guillem et al., 2006). Therefore, the mudpuppy is not resistant to TH, the TH/TR couple do not regulate any morphological change anymore but it still regulates the activity of other genes as in anurans.

3.3.2. Direct developers

More striking example of heterochrony than neoteny has been observed among amphibians. Direct developers are defined as species where young that hatch not as larvae (tadpoles in amphibians) from but as a miniaturized adult (Wolpert and Tickle, 2004) .

3.3.2.1. The (true) metamorphosis of *Eleutherodactylus coqui*

The common coqui *Eleutherodactylus coqui* is a peculiar frog since it is a froglet and not a tadpole that hatch from the egg. This tiny frog lives in the Central American rainforest trees and lays eggs with an unusual big yolk given its size (Callery et al., 2001). The ontogeny of *E. coqui* is strongly modified compared to amphibians with biphasic development. Within the egg, many larval characteristic are lacking, such as the coiled gut, the larval mouth and only the tail, which shrinks before hatching, is reminiscent of a tadpole (Callery and Elinson, 2000). Nevertheless, as in a regular metamorphosis TH pathway is involved in the late development of the frog. The TRs expression peaks at the remodeling climax, just before hatching, treatment with goitrogen prevents the remodeling, and this can be rescued by co-treatment of TH and goitrogens (Callery and Elinson, 2000). Interestingly, *in ovo* injection of CRH induces a precocious remodeling of the embryo, by stimulating the stress pathway, which in turn upregulates *TRβ* expression (Kulkarni et al., 2010). This is reminiscent of the *Xenopus* metamorphosis where the TH signaling pathway can be stimulated by stress factors. Together, the studies performed on *E. coqui* show that, although it is highly derived, its late development is homologous to the classical amphibian metamorphosis.

3.3.2.2. *Salamandra atra*

The alpine salamander *Salamandra atra* has not been subject of studies about metamorphosis but is known as an ovoviviparous salamander. The females do not lay eggs but carry its offspring (Greven, 1977, 1998). As a consequence, the mother does not give birth to water dependent salamander larvae but to a free-living juvenile (Greven, 1977). Unfortunately, no investigations of the role of TH on the *in utero* development have been performed. *Salamandra atra* is not an isolated example since salamander exhibit a wide variety of developmental strategies with or without clear metamorphosis (Denoël et al., 2005). This open the question of how often metamorphosis has been lost or displaced during the development of amphibian and how this is related to the history life trait of a species.

3.3.3. Smoltification in salmonids

Among teleosts, salmonids have a peculiar life cycle between fresh water and sea water. The eggs are laid in freshwater rivers. After hatching and growth, the parr spends several years in freshwater (Figure 17). When reaching a sufficient size, the parr undergo the smoltification that turns it into a smolt which is physiologically ready to enter seawater (Dittman and Quinn, 1996; McCormick and Saunders, 1987). This smoltification is considered by some authors as homologous to the amphibian metamorphosis as it involves physiological, behavioural and ecological switches, particularly the development of new pigmentation and the seawater acclimation. Nevertheless, this homology is still debated (Björnsson et al., 2012). TH level of the coho salmon *Oncorhynchus kisutch* rise during smoltification (Dickhoff et al., 1978; Young et al., 1989). In addition, treatments with TH induce a precocious smoltification and goitrogen treatment (propylthiouracil) delays this event (Sullivan et al., 1987). The expression of *dio2* increases in the brain prior to smoltification and at in peripheral organ such as the gills at the smoltification onset (Lorgen et al., 2015). Interestingly, the hormonal interplay occurring during freshwater to seawater transition involves a lot of hormones and not only TH. The growth hormone, insulin, the corticoid, but also the prolactin, which pathway counteract the TH signalling, are at stake to prepare the fish for the seawater (Folmar and Dickhoff, 1980; McCormick, 2001). All of these hormones are required for salmonids smoltification and TH does not act as a master hormone as in amphibian metamorphosis. This is why there is no consensus to consider smoltification as homologous to metamorphosis (Björnsson et al., 2012).

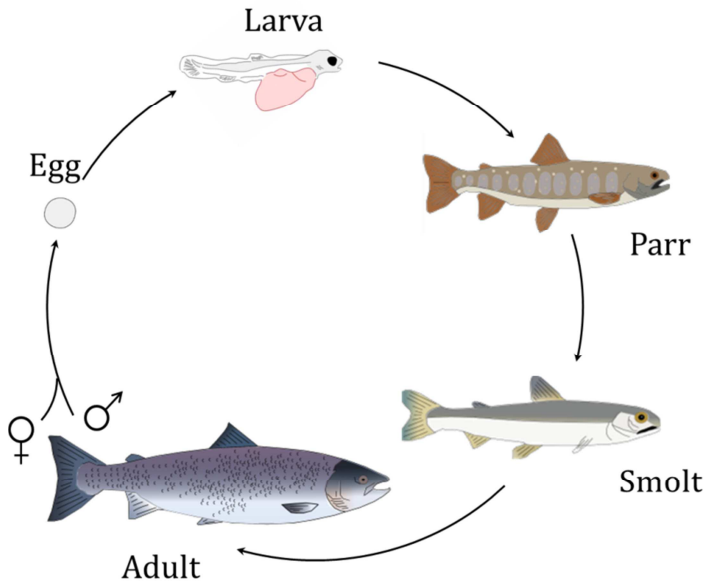

Figure 17: Life cycle of salmonids. Larva hatch from eggs and develops into Parrs. After several year in freshwater, parrs smoltify into smolt that are ready to enter seawater and develop as adults.

3.3.4. The (weird) metamorphosis of lampreys

Lampreys form with hagfish the most basal group of vertebrates and are therefore species of choice to study the evolution of vertebrates. The sea lamprey *Petromyzon marinus* has a biphasic life between river and ocean. The adults spawn into rivers, after hatching the benthic larva, called an

ammocoete, spend a few years in river as a filter feeder (Yousson, 1980). Eventually, the ammocoete moves toward the estuary to undergo its metamorphosis into an adult hematophagous ectoparasite. One interesting feature of lamprey metamorphosis from an evo-devo perspective is that the endostyle of the ammocoete (the mucus secreting area involved in filter feeder food trapping) develop in a true thyroid gland in the adult (Kluge et al., 2004). This is supported by histological and genetic data, particularly the expression of *ttf-1*, a gene required for thyroid development in vertebrates. As all vertebrates have a thyroid and non-vertebrate does not, lamprey is an interesting species to understand the evolution of the thyroid. Nevertheless, caution must be taken with the data from lamprey since its endocrinology might be highly derived (Thornton and Carroll, 2011). Other histological event reminiscent of the amphibian metamorphosis happened such as change in fat content (Kao et al., 1997) or switch of hematopoietic site (Percy and Potter, 1977).

If the lamprey metamorphosis is highly reminiscent of the smoltification of salmonids the endocrine control behind it is not and this is the most striking feature of lamprey metamorphosis. Indeed, instead of a peak, there is a global decrease of TH that is observed and that can induce the metamorphosis (Manzon and Youson, 1997; Youson et al., 1997). Pharmacological treatment with TH derivatives delays the metamorphosis and goitrogens can induce the lamprey metamorphosis (Manzon et al., 2001, 1998). Nevertheless, the mechanism of goitrogen action has not been investigated and we do not know how they work in lamprey. This situation of an inversed role of TH signalling on metamorphosis is the only one known, so far, in vertebrate. Surprisingly, the lamprey TRs are functional and are expressed throughout its metamorphosis (Manzon et al., 2014). The detailed mechanisms behind this peculiarity are unknown and the case of the lamprey metamorphosis is still an open question.

3.4. Metamorphosis of chordate: toward an unifying role of TH

Vertebrates are not the only animals undergoing metamorphosis as most of the species in other taxa have different phases of life separated by a development transition referred as metamorphosis. Interestingly TH and TR are involved in the metamorphosis of some of those species.

3.4.1. In Tunicates

Ciona intestinalis undergoes a metamorphosis during its life cycle. The free swimming non-feeding larvae settle down into a sessile feeding adult (Figure 18). Some experiments show that TH treatment on larvae accelerates, in some extend, the settlement and metamorphosis of the larva, but the effect is not as spectacular as in amphibians (Patricolo et al., 1981). The presence of T4 in *C. intestinalis* was assessed by immunodetection method (D'Agati and Cammarata, 2005) and there is a homolog of vertebrate deiodinase in another tunicate *Halocynthia roretzi* that is able to deiodinase T4 (Sheperdley et al., 2004). However, the TR of tunicates has not been demonstrated as functional. Thus, there is no evidence that the effects of TH on tunicate are mediated by the TR. As a consequence, in the state of our knowledge, we cannot conclude that the tunicates metamorphosis is homologous to the vertebrate metamorphosis as the couple TH/TR is not proven as functional It is not the hormone but the couple hormone/receptor that is require to prove the homology. *C. intestinalis* has a derived life cycle in comparison to other ascidians with a very short non-feeding larval stage. It is precisely this stage that end with a metamorphosis. As *C. intestinalis* development is

already happening fast, it might be complicated to further accelerate it with TH treatment. Moreover developmental mechanism in *C. intestinalis* might be highly derived too. Thus, *C. intestinalis* might not be the best model to understand the metamorphosis in tunicates.

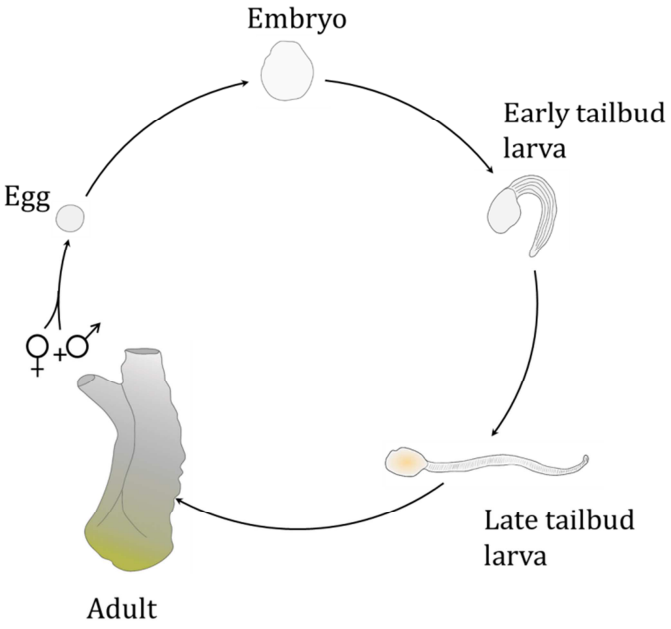

Figure 18: Life cycle of the sea squirt *Ciona intestinalis*. Embryos develop successively into early and late tailbud larvae!, This stage is very short in C; intestinalis since in last only a few days. Larvae then settle as adults. Adapted from Paris and Laudet, 2008.

3.4.2. In cephalochordates: the amphioxus *Branchiostoma lanceolatum*

Cephalochordates is the most basal taxon of chordates. Amphioxus (*Branchiostoma)* is a worm shape animal. The adults spawn into the water column and the embryos develop into pelagic asymmetric larvae with the mouth on the left and the gills slit on the right side. When it undergoes its metamorphosis, the animal settles to the sea bottom to become a benthic adult almost symmetrical (Figure 19; Bertrand and Escriva, 2011; Paris et al., 2008b). By the end of its metamorphosis, the amphioxus asymmetry disappears almost completely, the adult has a ventral mouth and gilt slits on each side of the body (Bertrand and Escriva, 2011)

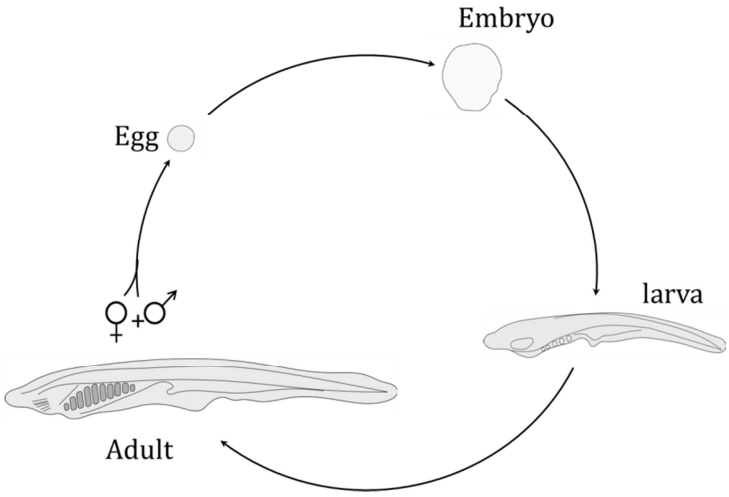

Figure 19: Life cycle of the amphioxus *Branchiostoma floridae*. Embryos develop into asymmetrical larvae that become symmetrical adult after their metamorphposis. Adapted from Paris and Laudet, 2008.

3.4.2.1. TRIAC controls metamorphosis in amphioxus

The study of amphioxus reveals its homology with vertebrate metamorphosis as it is controlled by TH signalling (Paris et al., 2008a, 2008b). The main difference though, is that the ligand of the amphioxus TR is not T4 or T3, but a derivative known as Triac (Paris et al., 2008a, 2010). Triac is a deaminated derivative of T3 (Wu et al., 2005) Amphioxus TR behaves as a *bona fide* TR: it binds DNA, binds Triac as a ligand and induces the transactivation of target genes when bound. Triac treatment of amphioxus larvae induces a precocious metamorphosis and treatment with an antagonist of TR delays it. Moreover TR expression peaks at metamorphosis and there is a TRE in the promoter of the TR (Paris et al., 2008a).

3.4.2.2. TH controlled metamorphosis appears at the basis of chordates

The amphioxus genome harbours some, but not all, homologs of the vertebrate genes involved in TH signalling (Paris et al., 2008b). It has a functional peroxidase oxidase involved that could have the same function of the vertebrate peroxidase involved in TH synthesis (Hiruta et al., 2005). Moreover, amphioxus has many deiodinases with a structure similar to the vertebrate one and one of them is specialized in Triac deiodination (Klootwijk et al., 2011). Together, these data build the hypothesis that the TH controlled metamorphosis appears at the basis of chordates (Paris and Laudet, 2008).

3.4.3. Ambulacraria

Ambulacrarias is the most basal group of Deuterostomes. It regroups two taxa, the echinoderms and the Hemichordates. Although closely related these two groups are very divergent in terms of body organization and development strategies as echinoderms often have a pentaradial symmetry and hemichordates a bilateral symmetry.

3.4.3.1. Echinoderms

Echinoderms is the most diverse group of the Ambulacraria, with many genus and species such as the sea star, the sea urchin, the sea cucumbers and the or sea cookie (or sand dollar). Our knowledge of the role of TH in the metamorphosis in echinoderm is reminiscent of the situation on tunicates. TH is found echinoderm tissues and accelerates the larva/juvenile transition as in the sea urchin *Lytechinus variegatus* (Heyland et al., 2006). Nevertheless, as in tunicate, the formal demonstration that the TR is functional and induces the transcription of target gene in presence of TH derivative has not been provided. Previous work performed in our team fails to show that the TR of *Strongylocentrotus purpuratus* binds any TR derivative (personal communication Mathilde Paris and Vincent Laudet). Without this, we cannot conclude that TH signalling is required and sufficient to induce metamorphosis as it is in the amphioxus and in vertebrates. In another sea urchin, *Strongylocentrotus purpuratus*, authors have shown that iodine uptake in the larvae is dependant of a peroxidase activity and they conclude that (i) there is an active accumulation of iodine in sea urchin larvae and (ii) that this process might be homologous to the accumulation of iodine in the vertebrates thyroid (Miller and Heyland, 2013).

3.4.3.2. Hemichordates

There are few studies interested into the late development of Hemichordates regarding the question of the metamorphosis evolution. The role of TH in their metamorphosis has not been directly assessed. In *Ptychodera flava*, a *ttf-1*(or *nk2.1*) homolog is found expressed in the pharyngeal structures but only during the juvenile development, not in embryogenesis (Takacs et al., 2002). *Ttf-1* is transcription factor expressed in the endostyle and is an early marker of the differentiation of the thyroid in vertebrates (Elsalini et al., 2003; Hiruta et al., 2005; Kluge et al., 2004). Finding this gene expressed in the pharyngeal region of a hemichordate is interesting in the perspective of the evolution of the metamorphosis control by TH. Nevertheless, TH has not been proven as involved in hemichordates metamorphosis. A TR has been identified in the genome of *Saccoglossus kowalevskii* but no functional experiments have been performed on it yet.

TH controls the metamorphosis of vertebrates, except in cartilaginous fishes and amniotes that are not considered as undergoing a metamorphosis From the data obtained in amphioxus we can conclude that TH controlled metamorphosis does also originate in the common ancestor of all chordates(Figure 20). We lack evidences to conclude that TH is directly involved in the metamorphosis of ambulacrarias through TR signalling.

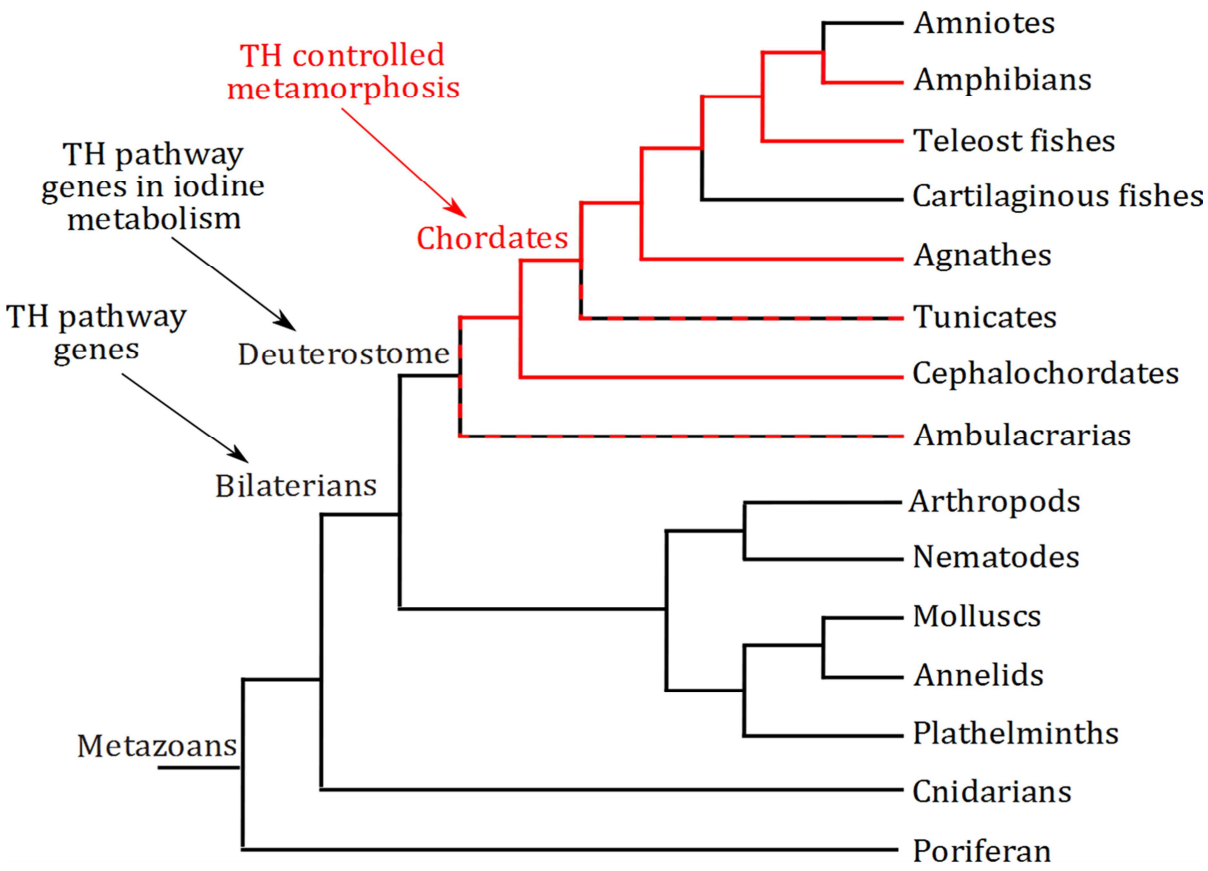

Figure 20: TH signaling in a simplified tree of metazoans. Red branches highlight taxa in which a TH controls metamorphosis. Dashed black and red branches highlight species in which TH might control metamorphosis, but the receptor has not been demonstrated as functional.

4. Project presentation

We investigated the role of TH in metamorphosis following two axes. First: how the timing of TH signalling impacts the metamorphosis and is integrated to environmental and ecological factors? We investigate this question the metamorphosis of the coral reef fish *Acanthurus triostegus*. Second: what is the origin of the TH signalling? We tackle this question by two means. The investigation of TH signalling in the annelid *Platynereis dumerilii* and the study of the origin of the Tg

4.1. Axis 1: Metamorphosis as a developmental event ecologically integrated

4.1.1. Metamorphosis of Coral reef fishes

As explained before, metamorphosis is influenced by environmental factors such as stress. We investigated metamorphosis using a coral reef fish model to take advantage of their life cycle.

Coral reef fishes have a biphasic life cycle. In most of the species, adult breed in the reef and eggs are carried in the open ocean by the water flow. The pelagic planktonic larvae develop into the open ocean. After this stage, larvae colonize the reef where they become juvenile. Those juvenile are recruited into the adult population and will reproduce (Leis and McComrick, 2002). The transition between the oceanic larvae into juveniles is referred as metamorphosis. Indeed, it implies abrupt morphological, physiological and ecological shifts (Leis and McComrick, 2002; McCormick et al., 2002), justifying designation of metamorphosis. Although the TH control of some teleost metamorphosis is well documented (de Jesus et al., 1998; Yamano et al., 1991), this is not the case in coral reef fish. Therefore we want to test the hypothesis that larval recruitment in coral reef fish corresponds to their metamorphosis regulated by TH and controlled by environmental clues. This will allow, to test if the framework developed in amphibians can be extended to these teleost fish.

Irrespective of having such a framework, it would be effectively important to better understand the factors that triggers larval recruitment. Indeed, the colonization of the coral reef fish through larval recruitment is critical to maintain a healthy adult population (Doherty, 2002). During their metamorphosis larvae, switch from the characteristics that enhance their survival in the open ocean (*e.g.* transparency, floating spine) to the ones that enhance their survival in the reef (*e.g.* camouflage, size). Colonization, which we define here as the entering of the larva in the reef, is a one night process and predation in the coral reef has an important effect on survival after colonization (Carr and Hixon, 1995). Thus it become clear that metamorphosis (*i.e.* changing) and colonization (*i.e.* moving) are the two side of the same coin. The success of this event is an important factor for fish survival (Doherty and Sale, 1985). Moreover, developing the features to detect the environmental clues is a prerequisite for the proper settlement of the juvenile in the reef until its recruitment in the adult population. Thus, metamorphosis can be seen as a process that determine the transition toward adulthood (Barth et al., 2015; Doherty, 2002)

4.1.2. The model: *Acanthurus triostegus*

To investigate coral reef fish metamorphosis, we used the convicted surgeon fish *Acanthurus triostegus* as a model. This teleost has a classical life cycle with a larval oceanic phase and reef phases as a juvenile and an adult (Figure 21A). It is abundant fish in French Polynesia and particularly in Moorea where our sampling come from thanks to our collaboration with the CRIOBE. The larval stage lasts between 44 and 60 days. During its metamorphosis, the planktonic feeder transparent larvae become pigmented grazer juveniles (McCormick, 1999). The overall morphology change (Figure 21B), the fish become less discoid and the mount move from a terminal to a ventral position (McCormick, 1999) The ectodermal appendages change since the thin vertical larval plates are replaced by the bigger adult scales (Frédérich *et al.*, 2010). Some interesting experiment of metamorphosis delay shows the link between metamorphosis and environment in the surgeonfish. Colonizing larvae were capture and the reef entry and displaced in a pelagic environment. Some individual delay their metamorphosis and retain their larval characteristic: transparency and morphology instead of developing as if they were in the reef. This indicates that the metamorphosis is a plastic event that can be influenced by environmental clues (McCormick, 1999). **We propose to characterize the metamorphosis of *Acanthurus triostegus* from a molecular perspective, in order to unify the endocrinological and ecological approaches and better understand the timing of coral reef fish metamorphosis.**

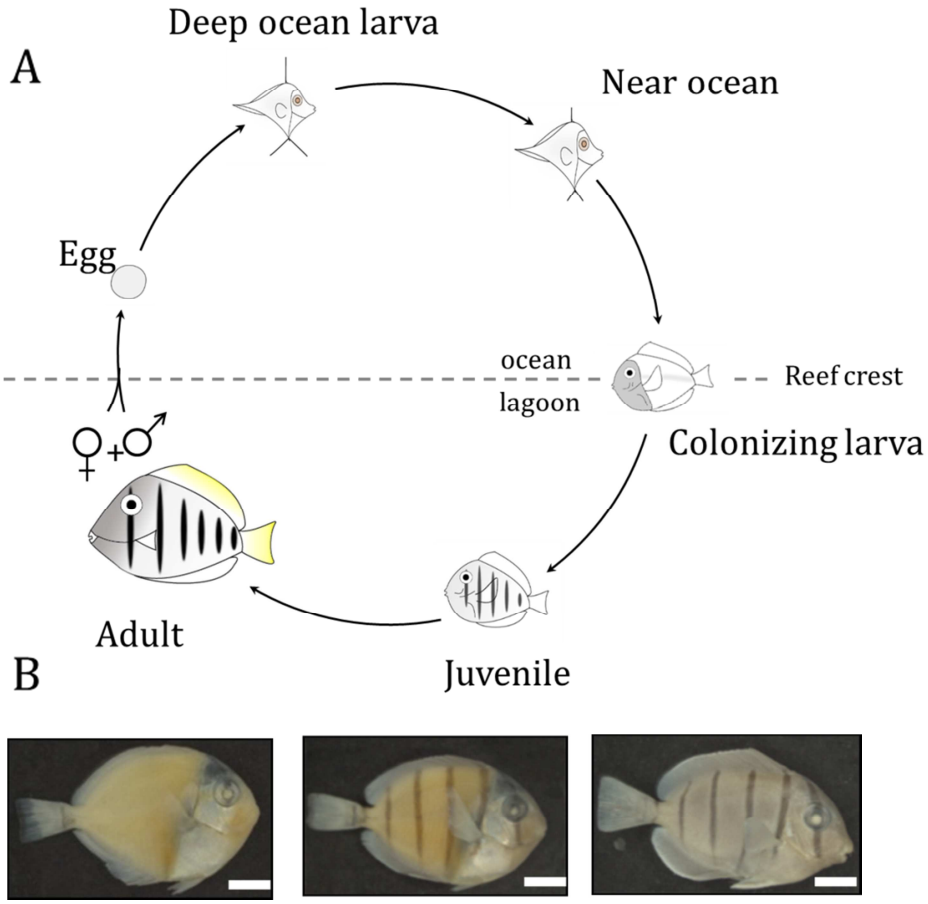

Figure 21: The convicted surgeaonfish *Acanthurus triostegus* A. Life cycle of *A.triostegus*. Adults reproduce in a reef pass and eggs are dispersed in the ocean. Larvae develop in the open ocean and eventually swim back to a reef after 44 to 60 days.. Colonizing larvae pass over the reef and settle as juvenile. Juveniles recruit into the adult population. **B.** Morphological change during the metamorphosis of *Acanthurus triostegus*. From left to right, fish captured at the reef crest, 1 day old juvenile and 9 days old juvenile.

Given that *A. triostegus* is a wild fish that cannot be breed in a laboratory and its biphasic life cycle, it worth explain the sampling method used in this study. All the sampling was performed by our collaborator in Moorea. Colonizing larvae were captured with crest nets. Those are fishing nest that are placed on the reef crest. When larvae colonize a reef, they surf on waves that go from the ocean, above the reef crest, into the lagoon. The nest intercepts those larvae. In order to have younger larvae, we use light trap. Those devices are positioned a few kilometres away from the reef. As fish larvae have a positive phototaxis, they are attracted and captured by the device. To obtain even younger larvae we used trawl haul to fish larvae of a few millimetres long at 25 to 40 meters depth 10 km away from the reef.

4.2. Axis 2a: Early TH signalling

4.2.1. Nuclear receptors signalling in bilaterian

Nuclear receptors are a vast superfamily of transcription factor and understanding its evolution and variation is important for two aspects.(i) This will allow to better understand the evolution of bilaterian, as the apparition of new function on pre-existing pathway (Markov and Laudet, 2011) or the regulation of organ development such as appendages growth (Gibert et al., 2015). (ii) Investigating nuclear receptors at a large scale and not only in mammals can help to unravel unexpected properties of nuclear receptors like the biding of non-canonical ligand (Navarrete-Ramírez et al., 2014) or the impact of endocrine disruptors on health and environment (Gunderson et al., 2001). Nevertheless, outside chordate, our knowledge of nuclear receptors signalling is scarce and mainly relies on identity of genomic data more than *in vitro* (or *in vivo*) experiments. Even if is gap is slowly bridged (Gutierrez-Mazariegos et al., 2014) our understanding of nuclear receptors signalling in protostome is far from being complete. Lots of efforts remain to have a clear view of the situation in protostome to securely infer the situation in urbilateria.

4.2.2. The model: *Platynereis dumerilii*

Platynereis dumerilii is an annelid (Lophotrochozoan, protostome) that emerged in the late 90's as a model in evo-devo. Classical protostomes models such as *Caenorhabditis elegans* or *Drosophila melanogaster* are all ecdysosoans. This induced a strong bias in evo-devo studies since it has been shown that these species have lost many genes during evolution. This is well examplified by NRs. As these species lose their TR (Bertrand *et al.*, 2004). Moreover, *P. dumerilii* has a slow evolving genome (Raible et al., 2005) and is thought to be representative, in some extend, of what urbilateria was (Zantke et al., 2014) .

P. dumerilii has a life cycle of several steps with transition referred as metamorphosis morphologically and ecologicaly described (Figure 23; Fischer et al., 2010). When adults mate, eggs fall at the bottom of the water column. After 24 hours post fertilization (hpf), the throchophore larvae emerges from the protective jelly and swim in the water column. Around 48 hpf the trochophore larvae start to lose its top-like shape and begins the transition toward the metatrochophore. During this transition the animal elongate by acquiring 3 primordial segments. Around 72 hpf the metatrochophore larvae become nectochates, they settle on the ground and start an exploring crawling, behaviour. This is the beginning of the settlement metamorphosis. At the end

of this settlement, around 120-168hpf, the larvae are called 3 segmented juveniles and feed. When it reached 5 segments, around 3-4 weeks old, larvae undergo the cephalic metamorphosis, acquire the definitive adult features, start to make protective tube and become more sedentary. After the cephalic metamorphosis, the atokous worms grow in width and length until the sexual metamorphosis that occurs several months later. At this step, worms stop feeding and turn into male or female (Fischer et al., 2010).

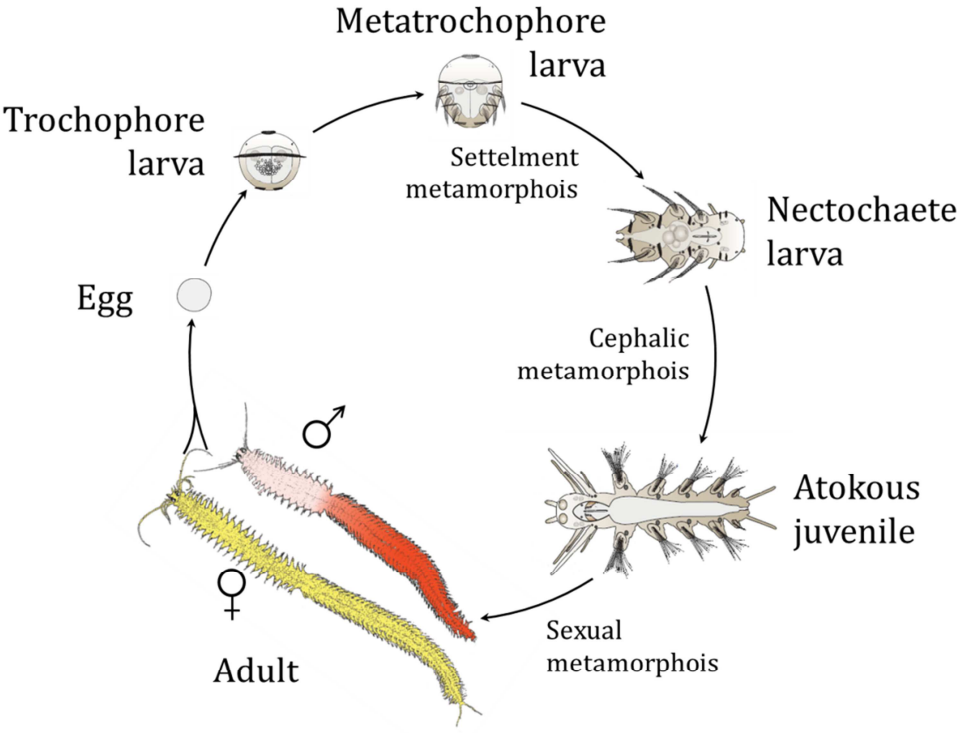

Figure 21:Life cycle of *Platynereis dumerilii*. Adults spawn eggs that develop into a trophophorous larvae. After a few days, these larvae settle down, to the floor and metamorphoses into a nectochaete larva. After a few weeks nectochate larvae undergo the cephalic metamorphosis an turn into atokous worms. Atokous worms undergo a posterior growth until the sexual metamorphosis when they turn into adults. Adapted from Fischer et al, 2011.

Thus, several step of *P. dumerilii* development are referred as metamorphosis as they correspond to spectacular life history transition. Indeed at each of these step, worms undergo behavioural (swimming/crawling/tubing), ecological (water column/benthos/benthos in tube) and morphological physiological (not eating/eating) changes. Each of this stage could be considered as a metamorphosis, similar in principle to chordate metamorphosis and therefore we asked in our work: (i) if TR signalling exist and is functional in *Platynereis dumerilii* and (ii) if it control one or several of these specific changes.

The existing literature provides very little information on the eventual hormonal regulation of these various metamorphosis. However, it is known that a neuropeptide signalling is involved in the settlement metamorphosis (Conzelmann et al., 2013). This neuroendocrine peptide is secreted by sensory neurone in the apical organ of the worm and trigger settlement behaviour through activation of a specific receptor in the apical organ too. This organ harbour many neurosensory neurons and this study show a mechanism by which environmental clues can be transduce in a behavioural response. Nevertheless, mechanism reminiscent of a systemic endocrinological control of metamorphosis have not been found. **We propose to characterize the TH signalling in *P. dumerilii* and to investigate its putative role in the development of the worm.**

4.3. Axis 2b: Tg as a vertebrate novelty: how did TH synthesis evolved?

4.3.1. TH synthesis knowledge comes from vertebrates

In vertebrate, TH is synthetized in the thyroid, a gland where iodine is concentrated and linked to the Tg. All our biochemical knowledge of TH synthesis comes from a limited number species in which Tg cDNAs have been cloned and the resulting protein characterized: mouse (Caturegli et al., 1997), rat (Kim et al., 2000) , human (Lamas et al., 1989), cow (Mercken et al., 1985), pig (direct submission) and dog (Lee et al., 2007). Those are the only animal for which Tg has been cloned. Thus, our knowledge of TH synthesis is strongly biased toward mammals? This is insufficient to understand the evolution of Tg at the scale of vertebrates. Moreover, mammals are homothermous and do not have a metamorphosis whereas amphibian and fishes are heterothermous and do have a metamorphosis. Body heat and metamorphosis are two characteristic controlled by TH. Consequently TH signalling in mammals on the one hand and amphibian and fish on the other hand is different and the synthesis of TH might have some specificity. As Tg is at the centre of TH synthesis it might harbour some differences too.

It is particularly striking that Tg is known in the two most important models outside mammals, namely the zebrafish *Danio rerio* and the clawed toad *Xenopus laevis* only by prediction from complete genome sequence of relatively poor quality. Indeed when scrutinized, the predictions of Tg in the genome of *Danio rerio* and *Xenopus tropicalis* appear to be incomplete or dubious and we have therefore no direct information of the precise mechanism of TH formation in these models.

4.3.2. Fill the gap in vertebrates: *Xenopus tropicalis* and *Danio rerio*

In order to have a better idea of the Tg biochemistry at the scale of vertebrates we cloned the Tg of *X. tropicalis* and *D. rerio* as model of the amphibian and teleost. Some partial fraction of Tg have already been cloned by other groups in those species and Tg is known for being expressed in the thyroid of both models (Regard and Mauchamp, 1971; Thisse et al., 2001). However, the predictions are not fully satisfying. The N-ter part of the protein that, in mammals, contains a peptide signal critical for the protein trafficking and maturation is not well predicted in fish and amphibian as well as the number of Tg repeat in each protein. **We propose to functionally characterize the Tg of *Xenopus tropicalis* and *Danio rerio*, focussing on the trafficking of the protein in order to have a broader view of the Tg evolution in vertebrates.**

II. Results

1. Larval recruitment in coral reef fish is a Thyroid Hormone controlled metamorphosis

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Abbreviated **title:** Thyroid hormone and metamorphosis of Coral fishes

Key words: Thyroid Hormones, Metamorphosis, Coral fishes, *Acanthurus triostegus*

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Abstract

Larval recruitment, the transformation and settlement of a pelagic larvae into a reef associated juvenile, is a key step for the maintenance of the adult coral reef fish populations. We investigated whether thyroid hormone (TH) that are known to control life history transitions in vertebrates coordinate the recruitment of a coral reef fish model, the convict surgeonfish, *Acanthurus trisotegus*. We show that the entry in the reef of larval *A. triostegus* correspond to a profound, morphological, physiological and ecological transformation. We observe that the settlement is coincident with a decrease of TH level, thyroid hormone receptor (TR) expression as well as a decreased expression of klf9 a classical TR target gene suggesting that the entry in the reef corresponds to the end of metamorphosis. We show that an increase of TH level and TR expression occurs early on in the deep ocean suggesting that an increase of TH signalling effectively trigger the whole process. Relocalisation of larvae on the reef external slope disturb, TH signalling, blocks the larval pigmentation change and freeze TR expression at levels that corresponds to younger larva. In addition, treating fish with a TR antagonist block the decreased expression of klf9 seen during reef entry. We also observed the changes of TH level observed in *Acanthurus triostegus* in unrelated species *Chromis viridis* and *Rhinecantus aculeatus*. Our data reveal that TH signalling effectively coordinate the recruitment of coral reef fish and provide a physiological framework to better understand the proximal mechanisms controlling this key life history transition.

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Introduction

Coral reefs are hotspot of biodiversity harbouring about 25% of the world's marine species (Mulhall, 2008) with a worldwide distribution. Nevertheless more and more evidences show that those habitats are endangered (Wilkinson, 2008). Most coral reef fish species exhibit a similar life cycle during which eggs are spawned in the reef and dispersed in the open ocean. Larvae develop in the ocean for several weeks or months, according to the species, and re-enter the reef at night. In the reef, newly entered larvae settle as juveniles, develop until they reach adulthood and reproduce to start the cycle again (Sale, 2002).

During this larval recruitment process, fish loose many of the characteristics that enhance survival in the plankton while developing other features suited to their new reef environment. This transformation is often called a metamorphosis (McCormick *et al.*, 2002). In this process, larvae undergo a high rate of mortality, particularly in the few days after colonization (Doherty & Sale, 1985). Indeed, failure of larvae to reach a suitable recruitment site results in an increased probability of death. This step is therefore critical for the maintenance of adult fish populations (Carr & Hixon, 1995). Several ultimate factors controlling this event have been identified. In particular, environmental clues such as odours or sounds are critical for the larva to localize and swim toward the reef, prior colonization, and to locate its habitat once in the reef (Barth *et al.*, 2015). In contrast the proximal factors that control the transformation of the larvae remain virtually unexplored with very few data about the time and space scale on which it occurs and little cues on what could trigger this phenomenon. Since the larval transformation is an ecological event coupled to a morphological transformation of the larvae into a juvenile we suspect it may be similar to the tadpole to frog metamorphosis well known in anourans amphibians.

In anourans it has been shown that it is a peak of thyroid hormones (TH) that triggers and coordinates metamorphosis (Brown & Cai, 2007). THs production is under the control of the hypothalamus-pituitary-thyroid (HPT) axis that controls the activity of the thyroid gland allowing an environmental coupling of the process. The thyroid gland produces mainly T4, the native hormone, with four iodine atoms. T4 is then activated in peripheral tissues by specific enzymes de deiodinase dio2 into T3, the active form, which contains only three iodine atoms (Chopra, 1996). THs levels peak at the climax of metamorphosis, when the morphological transformation of the larvae is maximal. Precocious treatment oh TH trigger metamorphosis and goitrogen (inhibitors of TH synthesis) treatment block it (Tata, 2006). TH signalling is mediated by the thyroid hormone receptors TRα and TRβ that are ligand-activated transcription factors belonging to the nuclear receptor superfamily (Laudet & Gronemeyer, 2001). During metamorphosis TRβ is self-induced through a positive feedback loop, therefore ensuring a precise coordination between the TH surge and the sensitivity of target tissues to the action of hormones (Machuca *et al.*, 1992, Shi et al., 1992). This allows the

regulation of a cell-specific gene regulatory network that coordinate the complex morphological and physiological remodeling of the tadpole.

The knowledge accumulated on *Xenopus* have been use to better understand other metamorphosis, in particular the transformation of the symmetrical flat fish larvae into an asymmetric juvenile (Yamano & Miwa, 1998; Yamano *et al.*, 2007; *et al.*, 2008; Schreiber, 2006). In these species also, TH levels and TRs expression increase at metamorphosis and consistently with amphibian model, goitrogen can prevent the transformation (McMenamin and Parichy 2013). However outside few species we have no general framework allowing comprehending the wide diversity of post-embryonic development strategies in teleost fish. We have proposed previously that metamorphosis regulated by thyroid hormone is an ancestral feature shared by all chordates (Paris et al., 2008; Laudet, 2011). This model implies that most if not all vertebrates, including teleost fish should display a larval remodelling period controlled by thyroid hormones. We believe that the larval recruitment of coral reef fish display all the feature of such an event and for this reason used the convict surgeonfish *Acanthurus triostegus* as a model to study the proximal causes underlying coral fish larval recruitment. This extremely common species exhibits a classic coral fish life cycle. Its transformation has already been morphologically described and corresponds to the reef colonization (McCromick *et al.* 2002, Frederich et al., 2012). Furthermore, it ecology is well known and there are evidences that environmental clues play an important role in *A. triostegus* metamorphosis (McCormick 1999, Frederich et al., 2012). In this study, we described *A. triostegus* metamorphosis using physiological, histological and morphological cues and investigate the link between the physiological event of the metamorphosis and the ecological event of reef recruitment. We demonstrated that the larval recruitment is effectively coordinated by THs and extend this observation to other unrelated coral reef fish species.

Material and Method

Fish capture, husbandry and conservation

Acanthurus triostegus larvae were captured at the Moorea atoll, French Polynesia. Larvae were captured at reef crest with crest nest during the night (Lecchini et al. 2004). Captured larvae were kept in water tank with non-filtrated lagoon water in open circuit. Larvae were fed with coral debris and algae turf up to the desired time.

A. triostegus near ocean larvae, *Chromis viridis* and *Rhinecanthus aculeatus* larvae were sampled at 1-2 km from the lagoon shore using light trap (Leis and McCormick 2002). Young *A. triostegus* larvae were also captured in the open ocean by trawl haul more than 10 km away from the reef between 25 and 40 meters depths. A very limited set of 6 larvae were captured with this technique.

Larvae were euthanized in MS222 at 0,4 mg/ml in seawater. For the TH dosage, larvae were dry frozen and conserved at -40°C (at Moorea) and -80°C (in Lyon) prior to extraction.. For histology, larvae were rinsed in PBS 1X , the intestine dissected and in Bouin's fixative at room temperature. For µCT scan and metagenomics, Larvae were kept in 70% ethanol at room temperature.

Cloning of *Acanthurus triostegus* genes

For RNA extraction, larvae were lacerated and kept in RNA later (Sigma) 1 hour on ice and stored at -20°C. Muscle from adult fish and whole crest captured larvae conserved in RNA later were used from RNA extraction. Sample were cut using a sterile scalpel blade and crushed in a Precelyss in a Qiagen extraction buffer. RNA extraction was performed using the Macherey-Nagel RNA extraction kit following the manufacturer.

Total pooled RNAs were retro-transcripted with the Invitrogen Super Script III enzyme following the manufacturer instructions. For TR cloning, actinopterygians universal primers (Table S1) were designed for TRα-A, TRα-B and TRβ to retrieve the full length sequence of the genes. For *polD2* and *rpl7*, actinopterygians universal primers were designed to retrieve partial sequence including at least one exon-exon junction. PCR amplicon were cloned in the Invitrogen PCR II plasmid following the manufacturer instruction for sequencing. TRα-A, TRα-B and TRβ were subcloned in pSG5 between EcoRI sites for *in cellulo* expression.

qPCR assay

For each larvae 1µg of RNA were used for retro-transcription using the Invitrogen Super Sript III following the manufacturer instruction, including a DNAse I treatment. qPCR primer were designed to anneal on different exons, *rpl7* and *pold2* were used gene for normalization (Table S2). qPCR were performed in 96 well plate with the BioRad IQ Syber Green Super Mix following the manufacturer proportion in 10µl of final reaction per well. qPCR were assayed on BioRad thermocyclers and analyzed on BioRad CFX Manager software. Assays were performed on duplicates in at least two independent RNA extractions and retro-transcriptions.

Phylogenetic analysis

The amino acid sequences of *A. triostegus* cloned TRs as well as available TR sequences (Table S3) were aligned using MUSCLE software (Edgar, 2004) Trees were generated using the Maximum Likelihood method (Saitou & Nei, 1987) and Seaview 4 software (Gouy *et al.*, 2010) under the JTT model with estimated gamma shape and eight rate categories. Bootstrap analysis of 1000 replicates was carried out to support the tree.

Functional characterization of the receptors

Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% of coal stripped foetal bovine serum and penicillin/streptomycin at 100µg/ml. Cell were maintained at 37°C, 5% CO_2. The transient transfection assays were carried out in 96-well plate with 30 000 cells per plate using Exgen500 according to the manufacturer instructions (Goula *et al.*, 1998). For each well, cell were transfected with 50ng of total DNA: 12,5ng of full-length receptor encoding plasmid, 12,5ng of reporter plasmid with four DR4 repeat in the luciferase promoter, 12,5ng of β-galactosidase encoding plasmid and 12,5ng of pSG5 empty plasmid. Drugs were incubated for 48 hours and cells were harvested using a passive lysis buffer and frozen at -20°C. On half of the lysate, luciferase activities were assayed with the luciferase reagent buffer from Promega on a Veritas Turner Biosystem luminometer. On the other half of the lysate, for normalization, the β-galactosidase activity was measured using ONPG substrate and absorbance at 420nm. Each assay was performed at least three times independently on well triplicates. Drugs from Sigma-Aldrich were diluted in DMSO à 10^{-2}M then in sterile PBS1X prior treatment.

Thyroid hormone quantification

THs were extracted from dry-frozen larvae as in Tagawa & Hirano, 1989 and Einarsdóttir *et al.*, 2006. Larvae were crushed with a Precelyss in 500µl methanol, centrifuged at 4°C and supernatant reserved, three times. Pooled supernatant were dried at 70°C. Hormones were re-extracted with 400µl methanol, 100µl chloroform and 100µl barbital buffer twice from the first dried extract. Pooled supernatant were dried out and extract were reconstituted in 1 ml PBS1X for. The quantification was performed following the Roche ELICA kit on a Cobas analyser by a medical laboratory according to the manufacturer standardized method.

Histology

The intestines in Bouin's fixative were embedded in paraffin. Sections of 7µm were performed using a microtome every 30µm. The histological sections were coloured Hematoxylin and eosin staining.

Gut Metagenomic

Crest larvae, 1 day juvenile, 9 days juvenile and adult *A. triostegus* gut were dissected in triplicates for a total of twelve samples. Total DNA was extracted a Macherey-Nagel DNA extraction kit following the manufacturer recommendations. 16S library were constructed for each of the individual using the Invitrogen 16S pipeline kit following the manufacturer recommendations. For crest larvae and 1 day juveniles, a 16S PCR amplification was performed because of the yield of bacterial DNA extracted were not suitable for library construction. Metagenomic sequencing of bacterial 16S ware performed using a PGM Ion Torrent. Three controls for contaminations were performed. The dissecting control consisted of an open tube during the guts dissection. The extraction control monitored the DNA extraction process. The PCR control, consisted in a blank PCR for the crest and 1 day samples 16S amplification. The sequencing results were then analysed using the Invitrogen 16S pipeline. The sequencing of the 18S was performed in a similar manner, using primer of the variable region V7. Two blocking primers were added to the amplification mix to prevent the amplification of the host DNA.

µCT scan analysis

Fish samples were conserved in 70% ethanol, dehydrated in successive baths of 95% ethanol, twice 100% ethanol and in vacuum arena for at least 4 hours. X-ray microtomographies were performed on a Phoenix Nanotom (General Electric) at 70kV of tension, 100 mA of intensity with a tungsten filament. 3000 images per sample were taken at 500ms of exposure per image and at a resolution of 2.5 to 2.8µm. 3D volumes were reconstructed and analysed with VGI studiomax software.

Results

Larval recruitment in *A. triostegus* is a complete transformation

During their entry into the reef the *A. triostegus* larvae undergo an extensive morphological remodelling (Fig. 1 A-E). Crest captured larva, as well as near ocean larvae have a typical pelagic pigmentation with a argentic shield (Fig. 1A). When these fish captured at midnight at day 0 are transferred in the lab, the next morning they exhibit a spectacular change in pigmentation with the appearance of vertical black stripes and a yellow background that will slowly shade in a few days (Fig 1B-E). The definitive juvenile pigmentation will be acquired at 5 days (Figure 1D) and the final morphology with the prominent mouth becoming more ventrally oriented near 8 days (Fig 1E) (Mc Cormick, 199; Frederich et al., 2012).

This external transformation is accompanied by internal changes that affect all studied organs. During the colonization, larvae weight a maximal value at the crest and then decrease with time suggesting that the settlement in the reef correspond to a real metabolic challenge (Fig. 1F). In accordance with this notion, the larvae intestine is surrounded by fat on crest-captured animals whereas on 1 day old larvae, these fat deposit disappear (data not shown). This metabolic challenge coincides with a change from a planktonic to algal-based diet revealed by previous isotope analysis (Frederich et al., 2012).

The change of diet should be accompanied by a coordinated change of the digestive tract. To further characterize the changes, we performed µCT scan and 3D reconstruction of *A triostegus* teeth from crest captured to 9 days old individuals (Fig. 1G-I). We observed three different families of teeth in the larvae, namely dental family A (red Fig. 1H), B (blue Fig. 1H-J) and C (green Fig. 1H-J) that replace one after another during the recruitment. Famiily A teeth are small, poorly mineralized and harbour 3 to 5 smooth tips. They are found of the side of the younger larvae. Family B teeth are medium-sized, mineralized with 7 tips on the mandible and 5 pointy tips on the maxillary. With their pointy tips, these teeth are typical carnivorous teeth. C-family teeth are much bigger, more mineralized and harbour a dozen of indentations on the upper jaw and 5 large and flat tips on the lower jaw, with a shovel-like form (Fig. 1I). These teeth are similar to the adult ones and exhibit typical grazer features. Teeth shift from A- to C-families are reminiscent of a change of diet during the colonization process from carnivorous to herbivorous. This shift is precisely organized since it happens from the median line to the distal part of the jaw with the central teeth erupting before the lateral ones (Fig. 1 H-J). Interestingly, there is some variations between individuals for teeth development since one near ocean larvae displayed C-family teeth more advanced than a crest captured larvae (Fig. S1).

The intestine also undergoes a drastic remodelling during colonization. The most striking event is a lengthening of the stomach-intestine apparatus from about 50mm (+/- 10 mm) in the crest-captured larvae to 790mm (+/- 100mm) in the 7-days old juvenile. To further characterize this change, we performed a histological survey of the intestine during the colonization process (Fig. 1 J-L). Because of the lengthening, we performed the intestine histology on the first centimetre, to compare equivalent areas. Intestine of crest captured larvae have a thick wall with many muscle fibre with close villosity and a small lumen (Fig. 1J). The size of the lumen increase and the villosity shrink in the few days following colonization (Fig. 1K-L). 8 days after colonization, the intestine wall is thin and the lumen wider (Fig. 1L). The hematoxylin and eosin staining reveal that the 8 days old villosities harbour more cell type than the crest villosities.

We perform a metagenomics sequencing of the bacterial community residing in the gut of *A. triostegus* comparing crest captured larvae, 1 and 9 days old juveniles and adult individuals (Fig. 1M). The 16S sequencing reveals a heat map of the most retrieved taxa of bacterial communities. The various controls group together and are different from any other sample. The controls contain taxa not observed in other samples such as the Caulobacterales, or in much higher proportion than other samples such as the Actinomycetales, thus validating the experimental procedure. The crest larvae and 1 days juveniles samples group together exhibiting specific taxa such as the Altermononadales or the Oceanospirillales, indicating that their bacterial communities are similar. In contrast, the 9 days juvenile samples exhibit a different profile with the appearance of the specific group of Stramenopiles, a higher yield of Vibrionales and a lower proportion of Alteromonadales than the crest and one days samples. These data show that the 9 days samples are undergoing a transition from the crest bacterial community toward the adult one. The progressiveness of this transition if highlighted by the fact that one of the crest larva sample groups with the 9 days juvenile samples. The differences in major bacterial group found in only 9 days reflect the speed and the magnitude of the ecological shift happening at colonization. The adult samples are the most dissimilar from the other ones. They harbour the highly specific group of Clostridiales and much less sequences from other taxa were found. This indicates that the bacterial community shift is not complete in early juveniles (9 days old) but keeps going on until adulthood, when individuals are known to graze different algal turfs than juveniles.

Taken together these results clearly show that the larval recruitment correspond to a complete remodelling of the animal in accordance with the notion that it corresponds to a classical metamorphosis.

The TH signalling is active during *A. triostegus* metamorphosis

The reef colonization by *A. triostegus* corresponds to it metamorphosis and TH signalling controls the metamorphosis of many teleost species. Thus, we investigate the TH signalling during the larval colonization of *A. triostegus*.

We aligned each TR of several teleost pecies in order to design degenerated primers to clone the *A. triostegus* TRs. We retrieve the three TRs of: *TRα-A*, *TRα-B* and *TRβ* that encode for putative proteins of respectively 410, 407 and 396 amino acids (Figure 2A). All the three sequences exhibit the TR classical domains: the A/B domain, the DBD domain, the hinge, the LBD domain and a small F domain. TRα-A has 86% of whole sequence identity with its *Danio rerio* orthologs, TRα-B a 96% of sequence identity and TRβ a 96% of sequence identity. As for all the teleosts, TR*β* has a long and short isoform with a difference of a nine amino acid in the LBD(Marchand *et al.*, 2004) .

We computer a phylogenetic tree of chordate TRs with available amino acid sequences (Table S3), the sequences we clone in this study and the *Branchiostoma floridae* TR sequence as outgroup (Figure 2B). The TRs of each species group with their respective orthologs, and the branch leading to TRα, and TRβ are supported both supported with 984 bootstrap replicates (out of 1000). The branch of the specific teleost whole genome duplication leading to TRα-A and TRα-B are supported with 601 and 825 bootstrap replicates respectively (out of 1000). The three *A triostegus* TRs group with their respective orthologs with the other teleost TRs.

We investigate the pharmacology of *A. triostegus* TRs to better characterize them. TRα-A, TRα-B and TRβ were cloned in the eukaryotic expression vector and transactivation of a Luciferase reporter was assayed by a transient transfection of HEK293T cells (Figure 2C). Thyroid hormone T4, T3, and their derivatives T2, Tetrac and Triac were tested from 10-9M to 10-7 M. The three TRs show a transactivation response for T4 at 10-7M but not below. The three TRs show a dose-dependent transactivation response for T3 and Triac from 10-9 to 10-7. TRα-B exhibits a weak but significant response for T2 at 10-7M. This is interesting to highlighted given that T2 is usually often as an inactive derivative of T3. No transactivation was obtained with Tetrac. These results show that the three TRs of *A. triostegus* acts as *bona fide* TRs.

To understand if THs have a role in *A. triostegus* metamorphosis, we investigate the level of total THs in far ocean larvae, near ocean larvae and reef colonization (Figure 2 D-E). We observed a drop of T4 by 3 fold between the near ocean captured larva and the 11 days old established juvenile (Figure 2D). The far ocean larvae have low level of T4. The situation of T3 is more complicated (Figure 2E). There was a huge variation of TH level within our three far ocean larvae. As those larvae were capture at a given distance of the reef, that is to say a geographical point, we cannot say if there were at the same

developmental stage, although they were morphologically similar. Near ocean and reef crest captured larvae have a similar high level of T3 that decrease once the fish is in the reef. Thus, T4 and T3 level of *A. triostegus* drop once the fish passed the reef. This is highly reminiscent of the end of the peak observed during amphibian metamorphosis, with the exception of the T3 level in far ocean larvae that remain surprisingly high. Interestingly, we observed some variation of the level of THs between individuals, at the same sampling point, especially the near ocean and crest individuals. As a result, there is an overall trend for a maximum THs level, for both T4 and T3, in near ocean/crest individuals and a subsequent decrease of THs level in older juveniles.

To fully characterise TH signalling at metamorphosis, we follow the expression level of *TRα-A*, *TRα-B*, *TRβ* and *klf9* (a TH controlled gene; (Denver & Williamson, 2009) during *A. triostegus* development (Figure 2F). On striking result is that far ocean larvae have two profiles, on with a very low expression of *TRs* and *klf9* and one with a very high level. Unfortunately, given the difficulty to capture these individual in the open ocean, we had only three individual to investigate which is not enough to conclude onb the level of expression of far ocean larvae. Aside from this sampling point, *TRα-A* and *TRα-B* exhibit similar expression profile between near ocean larvae and 8 days individuals. Indeed, expression of those TRs is higher in ocean than crests larvae than and juveniles. *TRβ* expression profile is slightly different with a peak of expression is observed in crest larvae and 1 days individual, as if the expression of this gene is shifted later in *A. triostegus* development. The expression profile of *klf9* is overall similar with a drop of expression between crest and 8 days individuals. Unfortunately, we were not able to obtain near ocean larvae to investigate *klf9* expression. These peaks are reminiscent of the peak observed in metamorphosis of amphibians and other fish species (McMenamin & Parichy, 2013). As for other fish species, the two *TRα* on the one hand and T*Rβ* on the other hand harbour different profiles.

TH levels coordinate the metamorphosis

In order to understand how TH signalling and the reef colonization , as an ecological event are linked, we disturbed the normal colonization of the reef by capturing larvae at reef crest and moving them to the external slope of the reef, as previously described (McCormick, 1999). We measure the level of T4 (Figure 3A) and T3 (Figure 3B) in fish captured at crest, 2 days old, 5 five days old and 8 days old in the reef, fish at similar age on the external slope without coral debris and fish at similar age on the external slope with coral debris. Fish on the external slope, with and without coral debris, have overall higher T4 and T3 level than fish of the same age in the reef. Their THs levels are similar to the THs level of crest captured fish.

To go further, we investigate the level of expression of the receptors and *klf9* when we manipulate the fish colonization (Figure 3C). As a result, the expression levels of the TRs are higher in the fish on the external slope than the fish in within the reef at the same age. This is consistent with the TH level. Interestingly, the fish exposed to coral debris on the external slope have a lower expression of TR than the fish without coral debris. This intermediate expression could indicate that coral debris, from the reef; attenuate the upregulation of TR caused by the capture on the external slope.

The TH-controlled metamorphosis is a conserved feature in coral reef fish

To going further in our hypothesis, we wanted to know if our observations in *A. triostegus* could be generalized to other coral fishes. Thus, we investigate the status of TH synthesis in the damselfish *Chromis viridis* and the triggerfish *Rinhecantus aculeatus* (Figure 4). Fish were captured in near ocean and keep in reef water with coral debris for 7 (*C. viridis*) to 9 days (*R. aculeatus*). For both *C. viridis* and *R.aculeatus*, we observe a drop of the TH level between the ocean captured larvae and the 7 days old individuals (Figure 4). The drop is clear in *C. viridis* for both T4 (Figure 4A) and T3 (Figure 4B) between near ocean captured and 7 days old fish. For *R.aculeatus,* the drop of T4 is clear between ocean and 9 days old fish (Figure 4C). However, there is a variation of the TH level of ocean captured fish which have similar level to the 1 day old fish. Then there is a drop of T3 with aging (Figure 4D). Thus, the decrease of TH observe in *A triostegus* and the synchronization with the recruitment to the reef can be generalized in other and distant coral fish species.

Discussion

Extensive morphological remodelling happens during *A. triostegus* colonization

In this study, we investigate the TH status of *A. triostegus* larvae during their recruitment to the reef. It is well establish that many teleost species undergo a TH-controlled metamorphosis during their development (McMenamin SK and Parichy 2013). Given that the metamorphosis of *A. triostegus* was morphologically descried during larval colonization, we investigate if this metamorphosis is also controlled by TH.

Accordingly with previous observation, *A. triostegus* undergo some profound morphological remodelling during metamorphosis (McCormick *et al.*, 2002). Given that there is a huge ecological shift and that *A. triostegus* change of diet at colonization (Frédérich *et al.*, 2012), we pushed the characterisation of the digestive tract changes further. We show that the teeth of the larvae change at colonization and switch from carnivorous-looking teeth to grazer-looking teeth. The teeth of *A. triostegus* are highly organized and we identified three families of teeth, ADT (A dental family), BDT and CDT that sequentially appears in the larvae. Interestingly, the CDF, which is the grazer-like teeth used by the juvenile in the reef, is already growing on the crest captured larvae. This indicates that the teeth growth is initiated before the reef colonization. In TH-controlled metamorphosis, intestine is an organ always targeted in the process, undergoing a deep remodelling under the hormone control (Schreiber *et al.*, 2005, 2009). *A. triostegus* is no exception since it intestine changes between crest larvae and juvenile reef fish. It becomes longer with a thinner wall and a wider lumen. Together, those data are consistent with a shift from a carnivorous diet to an herbivorous diet.

Accordingly with the change of diet, the bacteria community in the intestine changes too. It is known that feeding habit influences a lot the bacterial community of the host and recent study have shown that it is true for adults *Acanthuridae* (Miyake *et al.*, 2015). Moreover, it has been describe that the unusually large bacteria *Epulopiscium fishelsoni* is a commensal bacteria of *Acanthuridae* digestive tract (Angert *et al.*, 1993). This bacterium belongs to the clostridia taxa, which is the taxa we found the most represented in the adult *A. triostegus* gut. Interestingly, the 9 days old juvenile microbiota is not similar to the adult one. The Juvenile and adult *A. triostegus* do not live exactly the same microenvironment in the reef, the juvenile occupying smaller hiding places than the adult, thus the algae on which they feed are different too. This could explain why the microbiotas are different. Together, it show that the fish microbiota changes during the lifespan of the animal. Given the importance of microbiota in the health of an animal (Round & Mazmanian, 2009), bacteria gut

community is an interesting tool to assess the health of a population in relationship to the environment. Despite some recent efforts (Miyake et al., 2015), this kind of data still lack in fishes.

A. triostegus colonization is a TH controlled metamrophosis

Phylogenetic reconstruction of the three *A. triostegus* TRs and transactivation assay with several TR ligands indicate that *A. triostegus* TRs are functional and behave as genuine vertebrate TRs. Dosage of TH level during larval recruitment indicate that TH level drop between the crest and the 11 days old recruited juvenile. Given that a peak of TH level is observed at the metamorphosis of several teleost species we conclude that the same phenomenon happened in *A. triostegus*. The level of TH in fish correlates with the expression of the TRs and particularly *TRα-A* and *TRα-B* as their expression peaks when TH levels are the higher. Interestingly, *TRβ* expression peaks after, exhibiting a slightly different expression profile. Such a difference between *TRα-A* and *TRα*-B on the one hand and *TRβ* on the other is often observed during fish metamorphosis (McMenamin & Parichy, 2013). This also the case for the well-known amphibian metamorphosis, were *TRα* expression if high in early development and *TRβ* expression correlates with the peak of hormone (Eliceiri & Brown, 1994; Brown & Cai, 2007) . Indeed, in *Xenopus*, it is well establish that the two TRs have different role. TR*α* represses its target genes under low TH level before the onset of metamorphosis and TR*β* induces its target gene transcription at metamorphosis climax, ensuring coordination between the TH surge and the sensitivity of target tissues (Machuca *et al.*, 1995) . Moreover, the pharmacology of TRα-A, TRα-B and TRβ are slightly different. The expression profile of the gene *klf9*, follow the expression profile of the TRs and is consistent with the TH levels, indicating that TH signalling is effective in *A. triostegus*.

Far ocean larvae were capture in order to have an idea of the TH signalling status in early larvae before they are ready to colonise the reef. Surprisingly the T3 level in those larvae is high and highly variable whereas the T4 level is low. The TRs expression is also puzzling since two out of three larvae have a very low TR expression and one larva have a very high TR expression. This could indicate that there is a high variability of TH signalling or developmental advancement in far ocean larvae. Moreover, all these larvae were not sampled at the same geographical spot but at the same distance from the reef. Accordingly, larvae at a given distance might be differentially advanced in their development. This is already known for the colonizing larvae that have different absolute age (McCormick, 1999). The difficulty of having get access to these far ocean larvae is a major obstacle to solve the enigma of the TH signalling in early larva life of *A. triostegus*.

The external slope experiment shows that the normal TH signalling of the fish can be disturbed by environmental clues. Indeed, fishes that entered the reef but kept on the external slope have a crest-

like TH and TR expression profile, indicating that the normal decrease of TH and TR expression following the colonization can be hold on. This is consistent with a metamorphosis experiment performed by McCormick (McCormick, 1999) where he shows that *A. triostegus* kept on the external slope can delay their development.

Variability between individuals

We observe some variation between individuals captured at the same sampling point the TH level and the tooth development. Indeed some ocean-capture larvae exhibit lower of higher TH level than crest captured larvae. We have a similar observation for teeth, for which one near ocean captured larvae exhibit teeth development more advanced than crest-captured larvae. Thus, all the individuals are not synchronized when they colonise the reef. Given that *A. triostegus* decision to start colonization is under the control of environmental clues coming from the reef (Barth *et al.*, 2015), there must be a threshold at which the larvae begin their migration for the recruitment. Thus all the larvae might not have the same threshold. This highlights that metamorphosis is a plastic event and that all the individual do not dot develop exactly the same.

Generalization to coral fish species

To investigate if we could generalized those finding to other reef species, we investigate the TH level in the damselfish *Chromis viridis* and the triggerfish *Rhinecanthus aculeatus*. Ocean larvae were captured and grow up to 7 or 9 days, an age where they should be in the reef. The comparison of ocean captured TH level at the ocean stage and 7 days old indicate a drop of TH level for both species. As we observe a similar drop of TH as in *A. triostegus*, we hypothesized that *C. viridis* and *R. aculeatus* are also undergoing a TH-controlled metamorphosis just before their recruitment to the reef. This allows us to draw a global framework for metamorphosis and larval colonization in all coral fishes were both are tightly coordinated. This working hypothesis will allow us to better understand the connection between environment and physiology. This allows investigating the variations around metamorphosis and larval recruitment depending of the ecology of a given species, in a similar manner of the work performed on the direct developing frog *Eleutherodactylus coqui* (Callery & Elinson, 2000).

Conclusion

Together, TH level, TR expression level, histological and morphological changes indicate that the metamorphosis of *A. triostegus* is similar to the metamorphosis observed in other teleost and coordinated with the recruitment to the reef. Together these data exemplified coordination between the developmental event that is metamorphosis and the ecological event of the larvae recruitment. It immediately raises the question of how such coordination occurs and how it is controlled. Is it the onset of metamorphosis that triggers the recruitment or the contrary? It is known that stress is known to play a role in tadpole metamorphosis and controls TH synthesis in tadpole (Manzon & Denver, 2004) through the corticotropin-releasing factor and the HPT axis (Denver, 2009) . Moreover, it is known that environmental clues play an important role in the decision of the fish to actively swim toward the reef (Lecchini *et al.*, 2005; Barth *et al.*, 2015). TH controlled metamorphosis gives a framework to bridge the gap and understand the molecular mechanism between the ecological event of reef colonization and the physiological event of metamorphosis.

Figures and legends

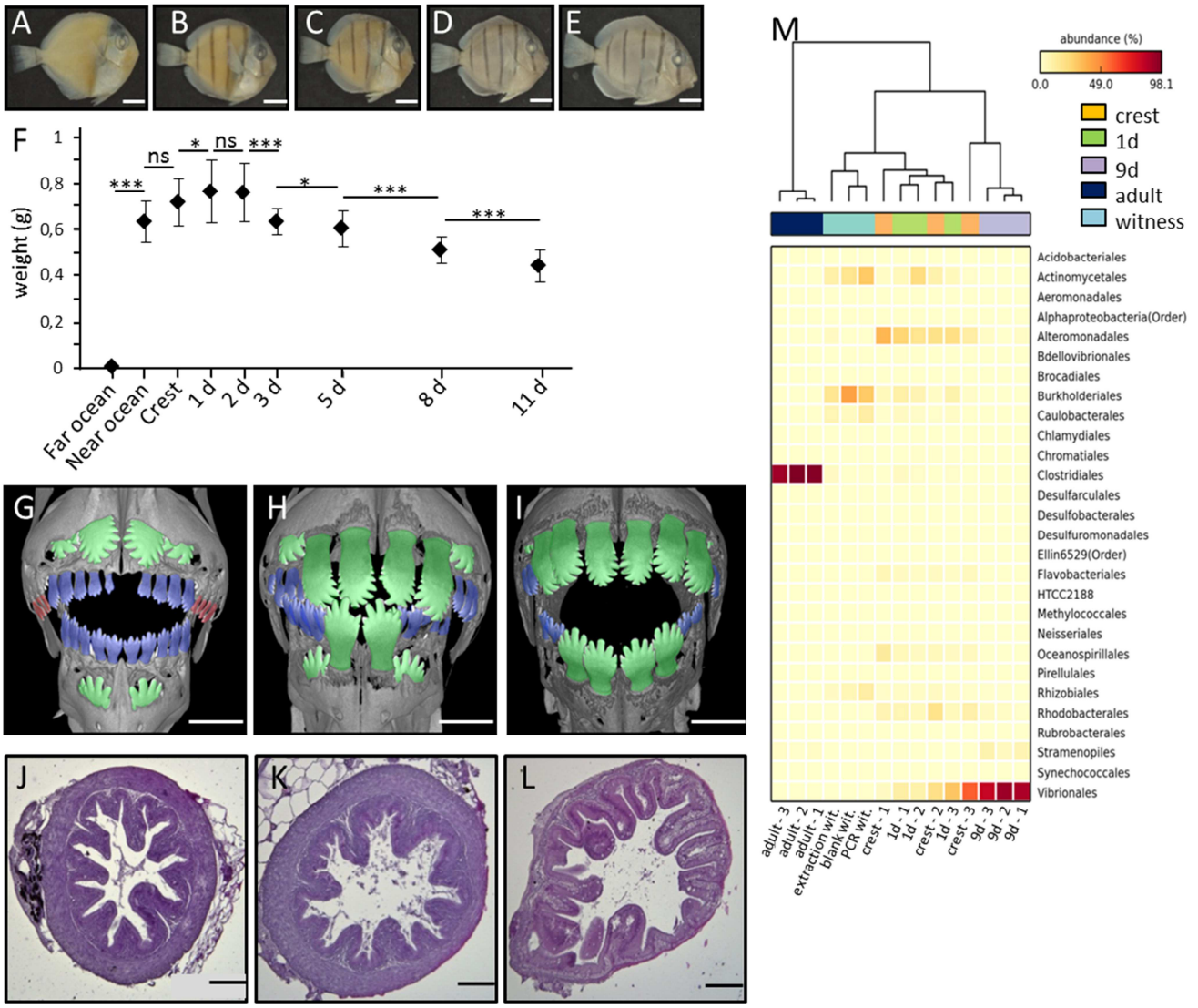

Figure 1: Changes in *Acanthrurus triostegus* during colonization /metamorphosis. (A-F) morphology of different sampling points, A: far ocean larva, B: crest larva, C: 1 day larva, D: 2 days larva, E: 5 days larva and F: 8 days larva. The scale bar indicates 0.5 cm. (G) Weight variation at colonization fish. Y-axis: weight in grams. X-axis: sampling point. Note that the far ocean, near ocean and crest points are not proportionally spaced. Student T-test: ns: non-significant difference; *: 0,05 > p-value > 0,01; ***: 0.005> p-value. (H-J) CT scan of larva: H: crest, I: 2 days old and J: 8 days old. ADF teeth are highlighted in red on H, BDF teeth in blue on H-J and CDF teeth in green on H-J. The scale bar indicates 0.5mm. (K-M) Histological section of intestine coloured by haematoxylin eosin staining of K: crest larva; L: 2 days larva and M: 8 days larva. The scale bar indicates 200µm. (N) Heatmap of bacteria taxa identified by 16S sequencing at various age. Each column is a sample and each rows a bacteria taxa. The sample names appear on the bottom line of the heatmap and on the upper line according to the following colour code: dark blue: adults, light blue: witnesses, orange: crest larvae, green: 1 day larvae and purple: 9 days larvae. The upper dendrogram indicates the order of similarity between the samples

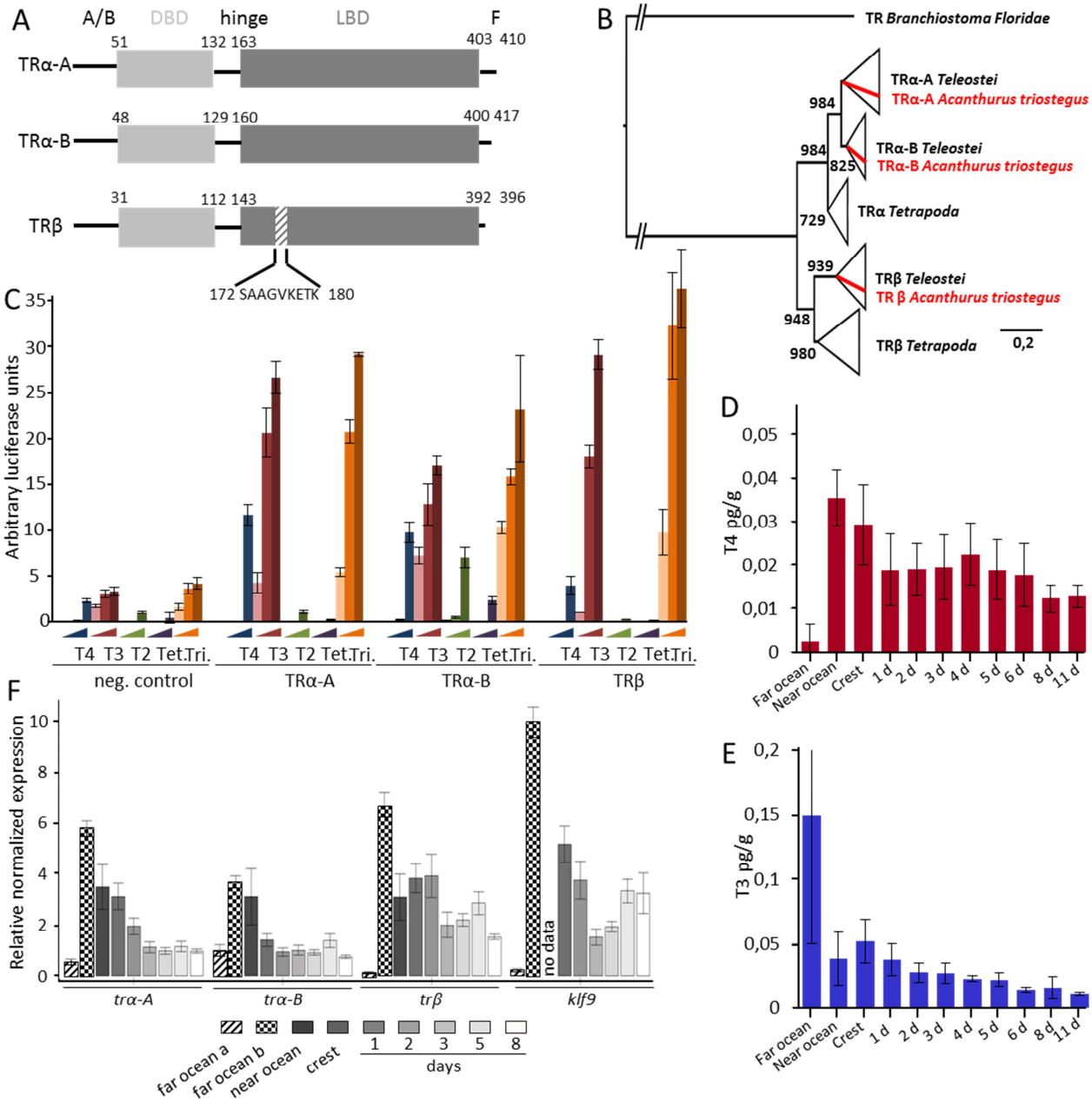

Figure 2: Thyroid hormone signalling during *Acanthurus triostegus* metamorphosis. (A) Representation TRα-A, TRα-B and TRβ respectively from upper to lower. The amino acid numbering of the DBD, light gray and LBD, dark grey, appear above each TR. The 9 amino acid insertion of the TRβ long isoform is indicated by grey stripe. (B) Phylogenetic reconstruction of TR amino acid sequenced by maximum likelihood. Bootstrap over supporting each branches are indicated when > 700/1000. *Acanthurus triostegus* genes are highlighted in red. (C) Transactivation assay of TRs in HEK293T cells with a luciferase reporter. Y-axis: arbitrary luciferase unit. X-axis Tested genes and treatment. blue bars: T4, red bars: T3, green bars: T2, purple bars: Tetrac and orange bars: Triac. The concentration range is 10^{-9}M to 10^{-7}M which correspond to the light and dark variation of each colour. Tested genes are indicated on the bottom line. (D) T4 dosage: Y-axis: T4 level in pg/p X-axis: sampling point. (E) T3 dosage: Y-axis: T3 level in pg/p X-axis: sampling point. (F) Expression level of *TRα-A, TRα-B, TRβ* and *klf9* by qPCR, normalized with *pold2* and *rpl7*. Y-axis; Relative normalized expression X-axis: gene, bottom line and sampling point. Far ocean larvae are splitted in two a: black stipes and b: black squares.

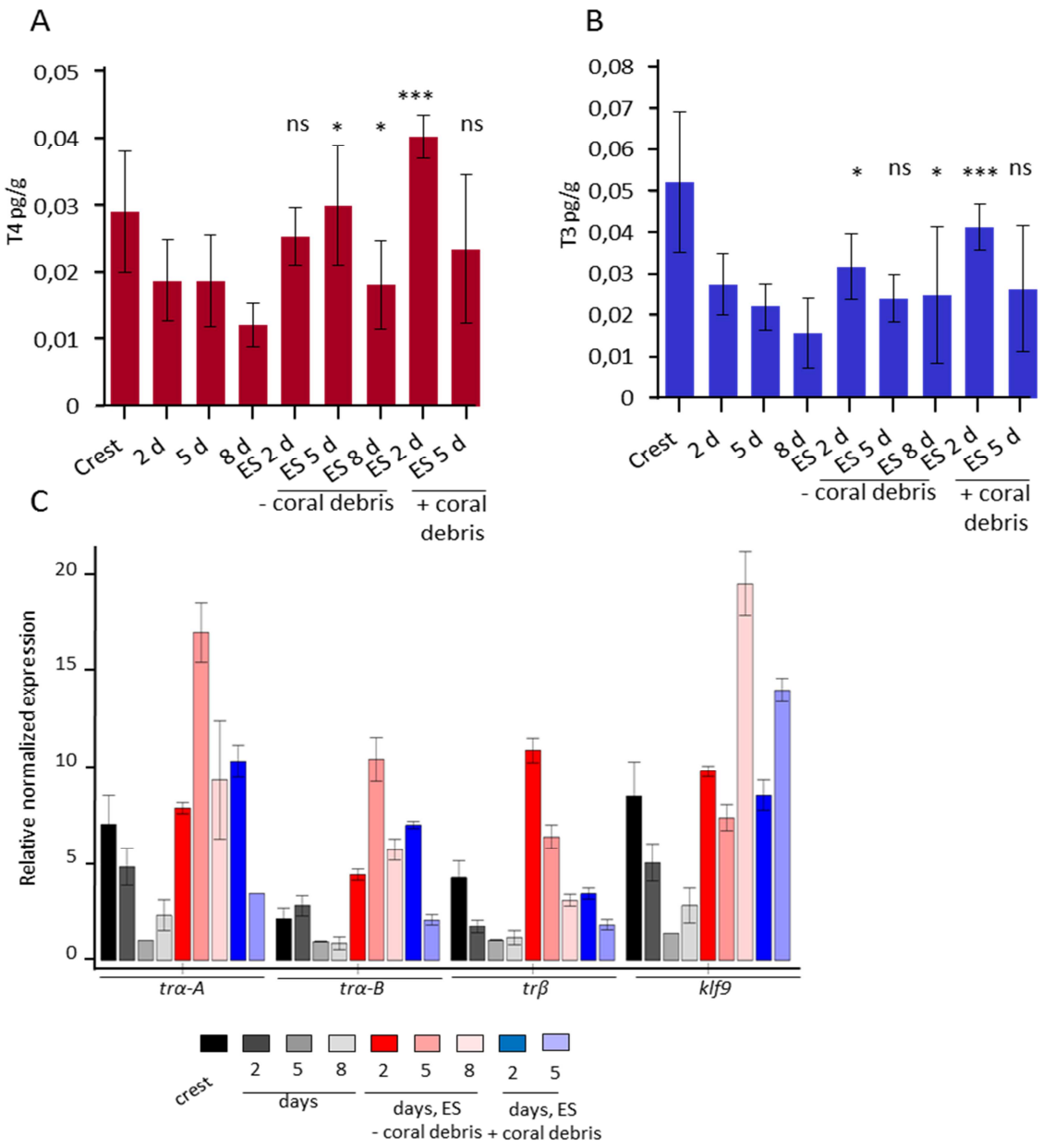

Figure 3: Manipulation of *Acanthurus triostegus* colonization and TH signalling. (A) T4 dosage of larvae at crest, 2 days old, 5 days old ans 8 days old, ES: external slope Student T-test: ns: non-significant difference; *: 0,05 > p-value > 0,01; ***: 0.005> p-value. With and withour coral ldebris condition is indicated on the bottom line. Y-axis: T4 level in pg/p X-axis: sampling point. (B) T3 dosage of larvae at crest, 2 days old, 5 days old ans 8 days old, ES: external slope Student T-test: ns: non-significant difference; *: 0,05 > p-value > 0,01; ***: 0.005> p-value. With and withour coral ldebris condition is indicated on the bottom line. Y-axis: T3 level in pg/p X-axis: sampling point. (F) Expression level of *TRα-A, TRα-B, TRβ* and *klf9* by qPCR, normalized with *pold2* and *rpl7*. Y-axis; Relative normalized expression X-axis: gene, bottom line and sampling point. Grey shades: normal colonization , red shades external slope without coral debris, blue shades: external slope with coral debris

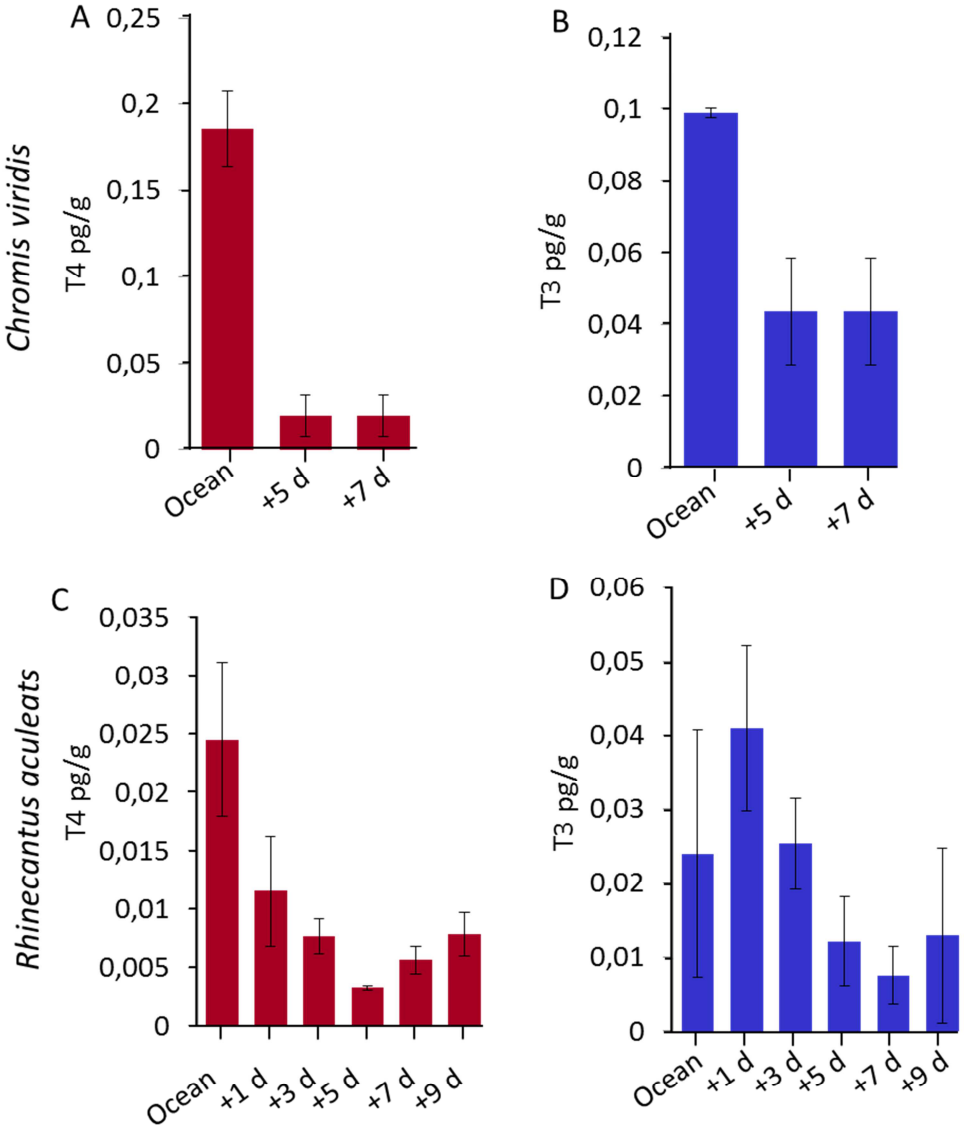

Figure 4: Thyroid hormone level of: *Chormis viridis*, (A): T4 and (B) T3 and: *Rinhecanthus aculeatus* (C): T4 and (D) T3. Y-axis: TH level in pg/p X-axis: sampling point.

Target gene	Sens	Sequence (5' to 3')	Annealing temperature
trα-A	F	CCTCAKTSTCCBGTGATTGGC	51°C
trα-A	R	TCABACYTCCTGGTCCTCRAASACC	56°C
trα-B	F	ATGGMACAMATGCCCRARGAGSAGG	56°C
trα-B	R	TCABACYTCCTGGTCCTCRAASACC	56°C
trβ	F	ATGTCAGAGCMAGSAGAVAAATGC	51°C
trβ	R	TCAGTCYTCRAASACYTCYAGGAAG	53°C
rpl7	F	CAGATCTTCAAYGGWGTNTTYGT	51°C
rpl7	R	GAADGGCCACAGGAAGTTGTTG	58°C
pold2	F	AGCTTCARYCGBCAGTATGC	51°C
pold2	R	CTGCTGTACYTCTVDATGTC	46°C
klf9	F	GVRCYHTRHRATGGYBGCVATGAT	60°C
klf9	R	TKVAKCATRCTKGGVTGGAAGYC	59°C

Table S1: Degenerated primer used for the cloning of the *A. triostegus* genes in this study. The indicated temperature is the mean temperature of the primers.

Target gene	Sens	Sequence (5' to 3')	Annealing temperature
trα-A	F	CAAAAGCCTCGCTCAGTTTAACCTG	60°C
trα-A	R	CTTGGCACTGCTCAATCTTCTCC	59°C
trα-B	F	GAAGAGGAAGAACAGCCAATGTTCG	60°C
trα-B	R	GTCACCACACACCACACATGG	60°C
trβ	F	GTCGCTGTCCTCCTTTAACCTGG	61°C
trβ	R	CCTCTTGGCAACGTTCGATTCG	60°C
rpl7	F	CAGGATTGCTGAGCCTTACATCG	59°C
rpl7	R	AATACGCTGTTTCCTCATCCTGC	60°C
pold2	F	CACCAAGAAGACTCAGGCTGG	59°C
pold2	R	GTGTAGTTGGTGGGGTCATACTGG	60°C
klf9	F	GCAAGTCGTCCCACCTCAAGG	59°C
klf9	R	GGAGAACTTCTTGCCACAGTCCG	61°C

Table S2: qPCR primer used in this study

Species	Protein	Accescion number	Database	Species	Protein	Accescion number	Database
Acanthopagrus schlegelii	TRα	ABQ96861.1	NCBI	*Monodelphis domestica*	TRα	NP_001184135.1	NCBI
Acanthopagrus schlegelii	TRβ	ABQ96862.1	NCBI	*Monodelphis domestica*	TRβ	XP_007505233.1	NCBI
Ambystoma mexicanum	TRα	AY174871.1	NCBI	*Mus musculus*	RARα	NP_033050.2	NCBI
Ambystoma mexicanum	TRβ	AY174872.1	NCBI	*Mus musculus*	TRα	NM_178060.3	NCBI
Anolis carolinensis	TRα	XP_008111558.1	NCBI	*Mus musculus*	TRβ	NM_001113417.1	NCBI
Anolis carolinensis	TRβ	XP_008117165.1	NCBI	*Necturus maculosus*	TRα	Y16623.2	NCBI
Branchiostoma floridae	TR	EF672344.1	NCBI	*Necturus maculosus*	TRβ	AY168331.1	NCBI
Canis lupus familiaris	TRα	NP_001273791.1	NCBI	*Oreochromis niloticus*	TRα-A	ENSONIT00000008144	Ensembl
Canis lupus familiaris	TRβ	XP_862690.2	NCBI	*Oreochromis niloticus*	TRα-B	ENSONIT00000022999	Ensembl
Conger myriaster	TRα-A	AB183396.1	NCBI	*Oreochromis niloticus*	TRβ	ENSONIT00000012974	Ensembl
Conger myriaster	TRα-B	AB183397.1	NCBI	*Oryzias latipes*	TRα	AB114860	NCBI
Conger myriaster	TRβ-A	AB183394.1	NCBI	*Oryzias latipes*	TRβ	AB114861	NCBI
Conger myriaster	TRβ-B	AB183395.1	NCBI	*Paralichthys olivaceus*	TRα-A	D16461.1	NCBI
Danio rerio	TRα-A	NM_131396.1	NCBI	*Paralichthys olivaceus*	TRα-B	D16462.1	NCBI
Danio rerio	TRα-B	XM_001920978	NCBI	*Paralichthys olivaceus*	TRβ	D45245.1	NCBI
Danio rerio	TRβ	NM_131340.1	NCBI	*Rattus norvegivus*	TRα	NP_001017960.1	NCBI
Epinephelus coioides	TRα-A	ABP62960.1	NCBI	*Rattus norvegivus*	TRβ	NM_012672	NCBI
Epinephelus coioides	TRα-B	ABP62961.1	NCBI	*Salmo salar*	TRα	NM_001123628	NCBI
Epinephelus coioides	TRβ	ABP62962.1	NCBI	*Salmo salar*	TRβ	NM_001123700	NCBI
Eublepharis macularius	TRα	AB204861	NCBI	*Sus scrofa*	TRα	NM_214190	NCBI
Eublepharis macularius	TRβ	AB204862	NCBI	*Sus scrofa*	TRβ	F1RS47_PIG	Ensembl
Gallus gallus	TRα	NM_205313.1	NCBI	*Xenopus laevis*	TRα	NP_001081595	NCBI
Gallus gallus	TRβ	NM_205447.1	NCBI	*Xenopus laevis*	TRβ	NP_001090182	NCBI
Homo sapiens	TRα	NM_199334.3	NCBI	*Xenopus tropicalis*	TRα	NM_001045796.1	NCBI
Homo sapiens	TRβ	NM_000461.4	NCBI	*Xenopus tropicalis*	TRβ	NM_001045805.1	NCBI

Table S3: List of TR sequences used for the phylogeny, species, gene, database and accession numbers are indicated.

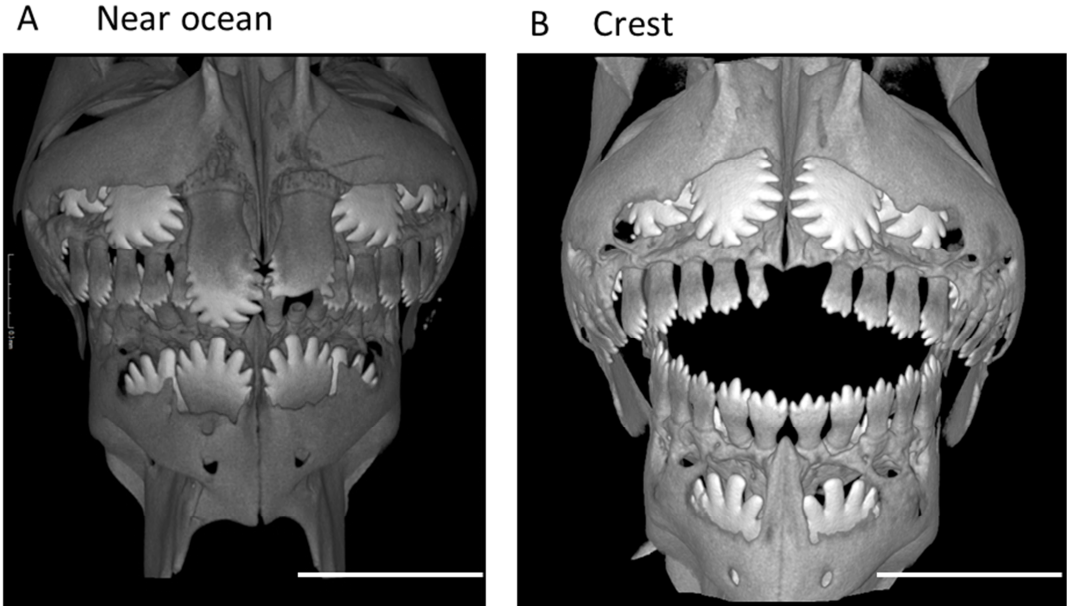

Figure S1: **CT Developmental disparity among colonising larvae.** Comparison of (A) Near ocean larva and (B) crest captures larva. The scale bar indicats 0.05mm

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2. *Platynereis dumerilii* enlightens the origin of thyroid hormone signalling

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Abstract

Thyroid hormones (THs) are multi-faceted hormones in vertebrates, playing a role in the control of post-embryonic life stages transition, energy metabolism as well as seasonality. It is established that TH signalling is functional in chordate but despite available genomic data, the situation remain unclear outside this taxa. We show that the TR of *Platynereis dumerilii* behaves like a genuine TR. It bonds DNA, several TH and derivative and is capable of transactivation, particularly with T3 and Triac. Pharmacological treatments show that T3 and Triac accelerate the late trochophore larval growth, the transition toward the morphologically bilaterian crawling larva. Given the phylogenetical position of *Platynereis dumerilii*, it suggests that TH signalling was functional in urbilateria and might be involved in development processes.

Introduction

Thyroid hormones (THs) are involved in pleiotropic processes in vertebrates as metabolic control (Mullur *et al.*, 2014), photoperiod signalling (Ikegami & Yoshimura, 2015) and metamorphosis (Tata, 2006). It is also known that THs are also involved in biological processes of non-vertebrate deuterostome. For instance, the metamorphosis of the chordate amphioxus is controlled by Triac, a TH derivative (Paris *et al.*, 2008). Moreover, the amphioxus genome exhibits several genes involved in TH pathway signalling (Paris, Escriva, *et al.*, 2008) which led to the conclusion that TH controlled metamorphosis happened in the common ancestor of all chordates. In Tunicates too, some evidences indicate a role for TH in development (D'Agati & Cammarata, 2005), but the demonstration of a functional TH receptor still lacks. It is the same framework in echinoderms since TH it can promote their metamorphosis, as in the sand dollar (Saito *et al.*, 1998), but no functional ligand of their receptor has been found. Moreover, in the purple sea urchin, there are data indicating a functional pathway for TH signalling (Heyland *et al.*, 2006). As a result, it is acknowledge that TH signalling pathway was functional, although its roles remain uncertain, in the common ancestors of all deuterostomes (Paris & Laudet, 2008). Genome sequencing predicted thyroid hormone receptors (TRs) orthologs in many of lophotrochozoans species (Bridgham *et al.*, 2010), but so far there is no evidence for a functional TH signalling pathway in any protostomes. Thus this paper, we investigate the case of the annelid *Platynereis dumerilii* to assess the question: is its TH signalling pathway functional?

Results and discussion

In order to better understand the thyroid hormone pathway in *P. dumerilii*, we investigate from the presence of orthologs of the TH pathway in *P.dumerilii* genomes (Figure S1). Aside from the TR, we find several genes exhibiting a clear orthology with vertebrate genes involved in the TH pathway: four deiodinase genes, one retinoid X receptor RXR. We also find other genes orthologs to several genes in vertebrate among which one is involved in TH pathway: Thyroperoxidase (TPO), Monocarboxylate transporter 10 (MCT10), thyrotropin-releasing hormone receptor (TRHR), Thyrotropin receptor (TSHR) (Figure S1). These finding indicate that a lot of genes of the TH pathway are present or putatively present in Platynereis.

P. dumerilii TR was cloned with primer within the UTRs to obtain the full length sequence of the receptor. The gene is 1320 nucleotides long and encodes for a 439 amino acids long protein exhibiting an overall of 42% identity with the human TRα: 25% for the A/B domain, 72% for the DBD and 42% for the LBD (Figure S2). The alignment with the human TRα and TR β proteins shows that the zinc fingers of the DBD are well conserved. In the LBD, four out of seven amino acids of the binding pocket are conserved. To have a better idea of its evolutionary rate, we construct a phylogeny of TRs (Figure1A). Although the phylogeny does not fully recapitulate the actual phylogeny of metazoans, all non-vertebrate taxa grouping as a consistent group, *P. dumerilii* TR groups with the other lophotrocozoans TRs without any particular long branch.

To further characterize the TR of *P.dumerilii*, we investigate its DNA biding, ligand biding and transactivation abilities (Figure 1B-D).We investigated the DNA biding ability of the TR by EMSA using several DNA probes that are classically bound by nuclear receptors. We found that *P.durmerilii* TR bound the classical HRE palindrome response element, as many nuclear receptors. Then, we investigate the binding to TR specific response element (Figure 1B). We found that it binds to DR4, the classical TRE, and in a lesser extend to DR3 and DR5. We did not found and any significant biding for DR0, DR1, DR2 or DR8. All these binding assays were also performed with a competitive cold probe which inhibited the binding of the labelled probe and a scrambled cold probe which did not. This indicates the specificity of the binding. Altogether, these assays show that *P.dumerilii* TR recognize and bound to the classical TRE.

We tested the ligand biding abilities of the TR by LPA using several potential ligand of the TR: T4, T3, T2, Tetrac and Triac at dose ranging from 10^{-9} to 10^{-7} M (Figure 1C). We found that T3 and Triac prevent the degradation of the receptor in a dose dependant manner, higher dose of hormone

derivate providing higher protection. This observation is also true in a lesser extent for T4, T2 and Tetrac. These results indicate that *P dumerilii* TR is able to specifically bind several derivatives of THs.

To assess the ability of the receptor to induce the transcription of target genes, we performed an *in vitro* transactivation assay. In HEK293T cells we transfected a Gal4(DBD)-TR(LBD) chimera constructs altogether with a UAS-Luc luciferase reporter construct. We test several potential ligand of the TR: T4, T3, T2, Tetrac and Triac at doses ranging from 10^{-10} to 10^{-7} M (Figure 1D). The *P dumerilii* TR transactivates in presence of T3 with doses as small as 10^{-10} M. T3 induces the transactivation of the receptor too starting from 10^{-9} M in a dose-dependent manner to 10^{-7}M. T2 behave as less potent agonist of the receptor, inducing a small transactivation at 10-7M. We did not observe any agonist activity for T4 or Tetrac at the tested doses. Going further, we test how the receptor behaves in presence of NH3, a pharmacological compound antagonist of vertebrates TRs (Lim *et al.* 2002, Nguyen *et al.* 2002). We co-treated cell transfected with our *P dumerilii* TR construct with T3 or Triac at a constant dose of 10^{-8}M with increasing dose of NH3 ranging from 10^{-10}M to 10^{-6}M (Figure 1E). As expected, the control NH3 alone does not show any transactivation by the receptor. When co-treating NH3 with T3, we were able to partially inhibit the transactivation of T3 with 10^{-8}M of NH3, that is to say a 1:1 ratio. We observe a strong inhibition by NH3 at a 1:10 ratio. Interestingly, we were not able to inhibit the transactivation of Triac with at a 1:10 ratio of NH3 but we at to treat to 1:100 ration of NH3. These results indicates (i) that NH3 acts as an antagonist of *P dumerilii* TR, (ii) together with the previous transactivation (Figure 1E) that the sensitivity to *P dumerilii* TR is much higher for Triac than T3.

In order to better understand the role of TH in the life cycle of *P. dumerilii*, we performed some pharmacological treatment at several stages of development with TH derivatives (figure 2A-E, table S1). Treatment with T3, Triac and in a lesser extend Tetrac at 6 or 12 hpf induces an acceleration of *P. dumerilii* development at 48 hpf with a more elongated larvae than the control. This shape is reminiscent of the shape of older animal although we do not know if this is due tu an elongation of the cell or an increase of cell division. As those treated larvae have the shape of older larvae, we propose that we induce an acceleration of the development by treating with TH derivatives. We do not observe this effect for T4 and T2 treatment (figure 2A, table S1A). Larvae treated at 18 hpf do not exhibit this development acceleration indicating a time window between 6 and 18 hpf where TH treatment is effective. NH3 treatment does not affect the development despite is effect on the receptor tested *in cellulo*. However, co-treatment of T3 with NH3 prevents the acceleration of development observed with T3 alone (figure 2B, table S1B). We do not observe such an inhibition

with Triac but it can easily be explained as transactivation assay show that Triac begin at a lower concentration than T3. This indicates that NH3 can act as an antagonist against an overdose of THs, as *in cellulo*. Interestingly co treatment of IOP, an inhibitor of deiodinase activity, with T3 or Triac also inhibits the acceleration of development observed at 48 hpf (figure 2C, table S1C) Although we did not investigate the direct effect of IOP on *P. dumerilii* deiodinases, this observation is consistent with an activating role of deiodinase which would be necessary to regulate the TH activity. In order to be complete, we also investigate the role of TH at the developmental steps described at metamorphosis in *P. dumerilii* development. Interestingly, this acceleration of development was not observed at 72 hpf and all treated larvae where similar in size and shape to the control condition (figure S3). Thus it is like if the T3 and Triac treated larvae have been cached up by the other larvae and that this step of development acts as a checkpoint. It is striking to observe an accelerating effect of TH at this stage of development since it is when the trochorphore larvae switch of morphology toward a bilateral symmetry.

We tested the settlement metamorphosis (figure 2D) occurring *ca.* 5-7 days which the transition between the late nectochaete and the three segmented errant juvenile and the cephalic metamorphosis (Figure 2E) *ca.* 1 month which is the transition between the errant juvenile and the atokous worms that undergo their terminal growth (Fischer *et al.*, 2010). We observe a slight increase of total length of the animal treated at 3 dpf and observed at 5 dpf but nothing that would indicate an acceleration of the larva toward the errant juvenile stage (figure 2D). When treated at 2 weeks, we did not observe any acceleration of the cephalic metamorphosis or any acceleration of the posterior growth (figure 2E). We also tested the sexual metamorphosis by treating 3 month old akotous worms with TH derivative and we did not observe any effect (data not shown).

The time window in which we observe an effect of TH derivative nicely correlates with the expression of the TR (figure 2F). Indeed, *P. dumerilii* expression peaks at 48 hpf and is much lower at the other stages of development. Conversely, the expression of the deiodinase we managed to clone is inversely correlated with the expression of the TR, with mow expression at the beginning of the development and high expression at later stages (figure 2G). This is consistent with a cross-talk of the different actirs of the TH signalling pathway in *P. dumerilii*. Together, those results are consistent with the idea of an accelerating effect of T3 and Triac specifically mediated by the receptor.

Our results show that TH signaling in *P. dumerilii* is functional and involved in its development,. *P. dumerilii* genome exhibits many genes involved in TH signaling including one TR that acts as a *bona fide* TR. This receptor binds DNA on TRE, it binds several TH derivatives and is able to transactivate a target gene in presence of TH derivatives. TH signaling is well established in chordate (REF) and several evidences show that it might be a synapomorphy of all deuterostome. Therefore, finding a

functional TH signaling in an annelid, member of the protostome branch indicated that it was already present in urbilateria. Thus it shows that TH signaling is a synapomorphy of all bilaterians and so, is much more ancient than anticipated (figure 3).

Interestingly, the transactivations assays show better gene activation for Triac than T3. Pharmacological treatments show that the observed effects of a Triac treatment are more resilient to the NH3 antagonism than the T3 treatment. Together, it indicates that Triac act as a better ligand than T3 for P. dumerilii. Moreover, in the chordate amphioxus the endogenous ligand is not T3 but Triac (Paris, Escriva, *et al.*, 2008) and a Triac specialized deiosinase has been identified (Klootwijk *et al.*, 2011) On the other hand, although Triac is known to activate vertebrate TR, it is believed to have a very short half-life and is not considered as an endogenous vertebrate ligand. Together, it asks the question of the ancestral ligand of TR in urbilateria and the view of T3 as the active ligand deriving from T4 light bias toward vertebrates.

The pharmacological treatments indicate that TH is likely involved in the development of *P. dumerilii* during at the trochophore to segmented juvenile transition step. The peak of TR at this step of development together with the possible acceleration of this step by TH treatment is reminiscent of the chordate TH-controlled metamorphosis since the same molecular actors are involved. Strikingly, the trochorphore to segmented juvenile transition period is particularly short in *P. dumerilii* when compared to other related annelids such as the *Malacoceros* genus. This situation is reminiscent of the case direct developer, organism in which a developmental period is shorter or absent compared to the related organism and TH controlled metamorphosis can be subject to such a direct development (Callery & Elinson, 2000) . If we consider *P. dumerilii* development as already accelerated, it might explain why we lose the effect of our TH treatment at 72 hpf since we are not able to accelerate a process that is already fast.

Having a functional TH signaling also raises the question of the TH synthesis since no ortholog of the thyroglobulin (Tg), the central protein involved in TH synthesis in vertebrate (van de Graaf *et al.*, 2001) has been found. This is consistent with our current view of the evolution of this protein, even if the domains forming this protein are found in protostome; it is not enough to claim an orthology of Tg with any prtotosome protein (Novinec *et al.*, 2006) This is also true for the amphioxus, a TH signaling without Tg. Unfortunately, we lack a conceptual framework to understand how to produce TH without Tg and thyroid since most of our data about TH synthesis come from vertebrate studies. Hence there are two possibilities, an endogenous or an exogenous source of TH. The endogenous hypothesis is that TH can be synthesized from another protein than Tg. We suggest than the pharyngeal region could be where this synthesis happened. As for the vertebrate thyroid, the non-vertebrate endostyle is made of glycoprotein complexes. The endostyle of lamprey is homologous to

the vertebrate thyroid (Kluge *et al.*, 2004; Hiruta *et al.*, 2005) and iodine compounds are found in the cuticle and the pharynx of annelids near the pharyngeal teeth(Gorbman *et al.*, 1954) It is an old hypothesis that invertebrate are able of a true TH metabolism (Gorbman, 1955) and finding a functional TR in annelid gives a molecular support to this hypothesis. The alternate hypothesis is the exogenous source of TH which might come from food since some authors have found iodine derivative compounds in some algae (Roche & Lafon, 1949; Edmonds & Morita, 1998) although it remains controversial (Edmonds & Morita, 1998).

Material and Method

Cloning of *P.dumerilii tr* and thyroid hormone related genes

Whole RNA from 125 hpf *P.dumerilii* larvae were isolated in Trizol:Chloroform at 5:1 from the aqueous phase, precipitated in isopropanol and subsequently purified on a RNAeasy Qiagen column following the manufacturer instruction. Retrostranscription was performed using the Super Script III from Invitrogen following the manufacturer instruction. Primer form *tr dio*, *tpo*, and *mct10* orthologs cloning were design based on transcriptomic data available in Arendt lab (table S2). TR primers were designed in the UTRs to get the full sequence and Dio primers were designed within the ORF. Cloning was performed on cDNA using a Phusion Hot start II High Fidelity polymerase from Thermo Scientific following the manufacturer instruction. Full length *TR* was cloned in pSG5 between two BamHI sites. The LBD of *TR* was cloning in pG4M plasmid, a pSG5 backbone with a Gal4(DBD), in frame between XhoI and BamHI restriction sites. *Dio* was cloned and in pCRII in TOPO cloning vector from Invitrogen following the manufacturer instructions.

qPCR assay

P.dumerilii were distrupted in Trizol:Chloroform 5:1 solution at stages 24 hpf to sexually differentiated adults. Aqueous supernatants were proceeded into Macherey-Nagel RNA extraction column following the manufacturer instructions. For each individual 1µg of RNA were used for retro-transcription using the Invitrogen Super Sript III following the manufacturer instruction, including a DNAse I treatment. qPCR primer were designed to anneal on different exons, *HSP70, Rsp9, Cdc5* (Dray *et al.*, 2010) and *α-tubulin* (Altincicek & Vilcinskas, 2007) were used gene for normalization (table S3). qPCR were performed in 96 well plate with the BioRad IQ Syber Green Super Mix following the manufacturer instruction reduced to 10µl of final reaction per well. qPCR were assayed on BioRad thermocyclers and analysed on BioRad CFX Manager software. Assays were performed on duplicates in at least two independent RNA extractions and retro-transcriptions.

Phylogenetic analysis

To reconstruct the phylogeny of the, we concatenated the DBD and LBD amino acid sequences. We used the sequences of *P.dumerilii* cloned in sthis study altogether with TR sequences retrieved in various databases (table S4). Sequences were aligned using MUSCLE software (Edgar, 2004). Trees were generated using the maximum likelihood method (Saitou & Nei, 1987) and Seaview 4 software

(Gouy *et al.*, 2010) under the JTT model with estimated gamma shape and eight rate categories. SH-like support was computed to assess the branches strength.

Electrophoresis Mobility Shift Assay (EMSA)

EMSA were performed using full length amphioxus NR7 and RXR protein cloned in pSG5 vector and synthetized using the TnT coupled reticulocyte lysate system from Promega following the manufacturer recommendations. Binding were performed in a Tris pH 7.5 buffer at room temperature. Running was performed in a 5% polyacrylamide 40:1 gel in TBE buffer. DR0 to DR8 Probes were labelled with ATP-γ32P using Promega kinase following the manufacturer recommendation (Table S5).10 to 100 fold excess of non-labelled probes were used in as specific competitors. Scramble probe was used a non-specific competitor. Exposition was performed with on phosphorimager screen and read with a Typhoon phosphorimager from GE Healthcare.

Limited Proteolysis Assay (LPA)

P dumerilii TR, RXR and rat TR, RXR full length protein were synthetized with ^{35}S methionine using the TnT coupled Reticulocyte Lysate System from Promega following the manufacturer instructions. Incubation with trypsine was performed at room temperature10 minutes with ligand concentration from 10^{-9} to 10^{-7} M. Migration was performed in 12% polyacrylamide 37.5:1 gel in 10% SDS Tris buffer. Exposition was performed with on phosphorimager screen and read with a Typhoon phosphorimager from GE Healthcare.

Transactivation assay

Human embryonic kidney 293 T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% of coal stripped foetal bovine serum and penicillin/streptomycin at 100 g/ml. Cell were maintained at 37°C, 5% CO_2. The transient transfection assays were carried out in 96-well plate with 30 000 cells per plate using Exgen500 according to the manufacturer instructions. (Goula *et al.*, 1998). For each well, cell were transfected with 50ng of total DNA: 12,5ng of Gal4-LDB receptor encoding plasmid, 12,5ng of reporter plasmid with an UAS response element in the luciferase promoter, 12,5ng of β-galactosidase encoding plasmid and 12,5ng of pSG5 empty plasmid. Drugs were incubated for 48 hours and cells were harvested using a passive lysis buffer and frozen at -20°C. On half of the lysate, luciferase activities were assayed with the luciferase

reagent buffer from Promega on a Veritas Turner Biosystem luminometer. On the other half of the lysate, for normalization, the β-galactosidase activity was measured using ONPG substrate and absorbance at 420nm. Each assay was performed at least three times independently on well triplicates. Drugs: T3, T4, T2, Tetrac, Triac from Sigma-Aldricht were diluted in DMSO à 10^{-2}M then in sterile PBS1X prior treatment. The NH3 drug was ordered from AVG discovery.

Figures and legends

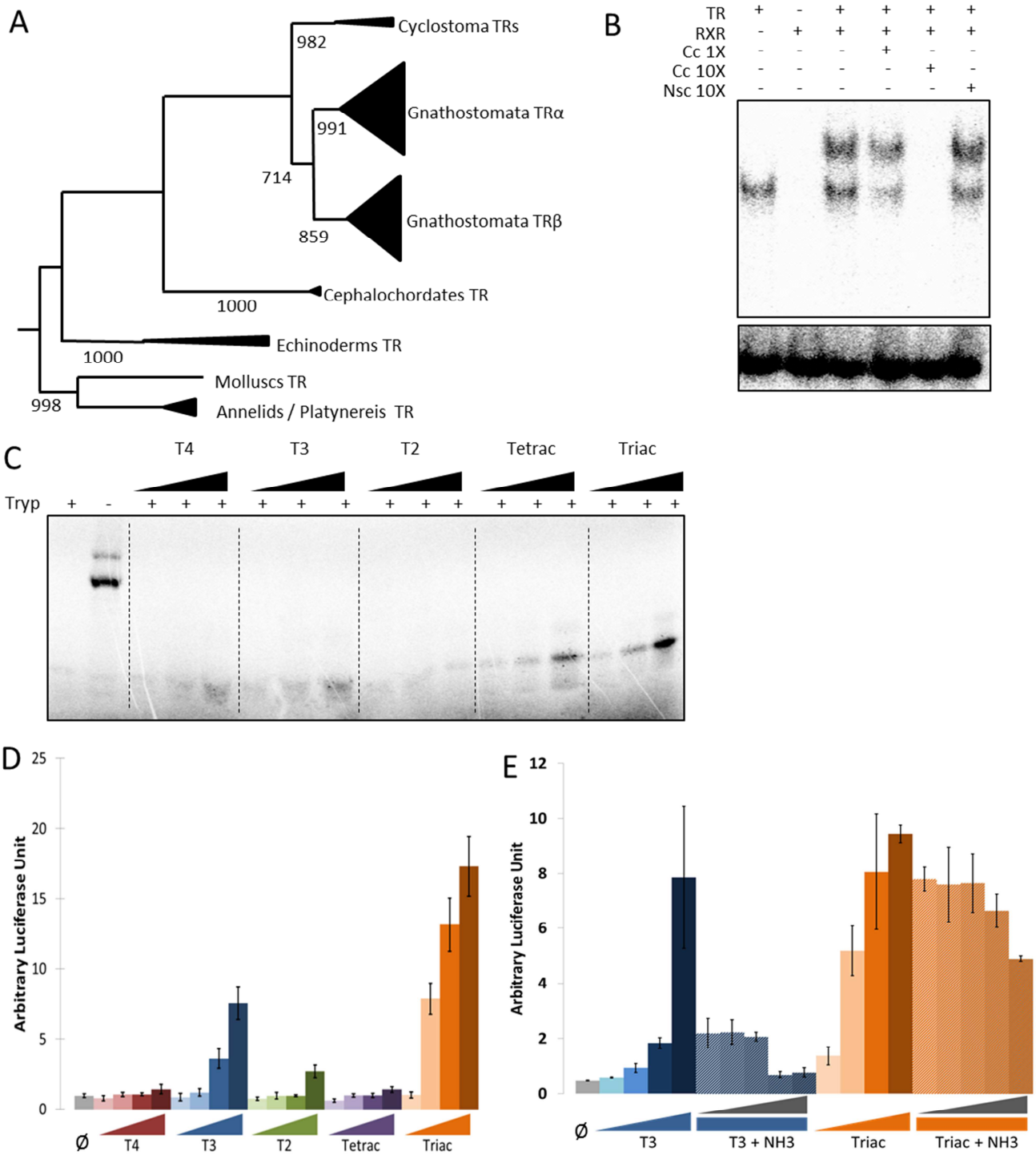


Figure 1. *Platynereis dumerilii* TR is a genuine TR (A) Phylogenetic tree build on protein sequence by maximum likelihood of *P. dumerilii* TR and other bilaterians TR. Bootstrap support over 700 out of 1000 replicate are indicated. (B) *In vitro* Electro Mobility Shift Assay of *P. dumerilii* TR with a ^{32}P labelled TRE probe. Cc1x: Non-labelled cold competitor probe at a concentration of 1X the labelled probe. Cc10x: Non-labelled cold competitor probe at a concentration of 10X the labelled probe. Nsc: Non-specific competitor of random sequence at a concentration of 10X the labelled probe. Band shift are on the upper panel and excess of probe are on the lower panel. (C) *In vitro* Limited Proteolysis Assay of *P. dumerilii* TR with an increase amount of T4, T3, T2, Tetrac and Triac as TH derivative. Amount of ligand are ranging from 10^{-8} to 10^{-6} M of final incubating concentration. The black triangles indicate the increasing concentration. (D) Transactivation assay in HEK293T cells of *P. dumerilii* TR with a luciferase reporter plasmid. Arbitrary unit of luciferase activity are labelled on the Y axis. Grey bar labelled Ø indicates the control condition with no treatment. Red bars are for T4, Blue bars for T3, green bars for T2, purple bars for Tetrac and orange bars for Triac. The triangles indicate the increasing concentration together with the darkening colors. The concentrations range from 10^{-10} M to 10^{-7} M. Error bars correspond to the standard deviation of measure performed in triplicate. (E) Transactivation assay in HEK293T cells of *P. dumerilii* TR with a luciferase reporter plasmid. Arbitrary unit of luciferase activity are labelled on the Y axis. Grey bar labelled Ø indicates the control condition with no treatment. The blue and orange triangles respectively indicate treatment with T3 and Triac from 10^{-10} to 10^{-7} M. The gray triangles with blue and orange rectangles respectively indicate co-treatment of T3 + NH3 and Triac + NH3. T3 and Triac was fixed at 10-8 M in all the co-treatment condiction and NH3 are ranging from 10^{-10} M to 10^{-6} M. . Error bars correspond to the standard deviation of measure performed in triplicate.

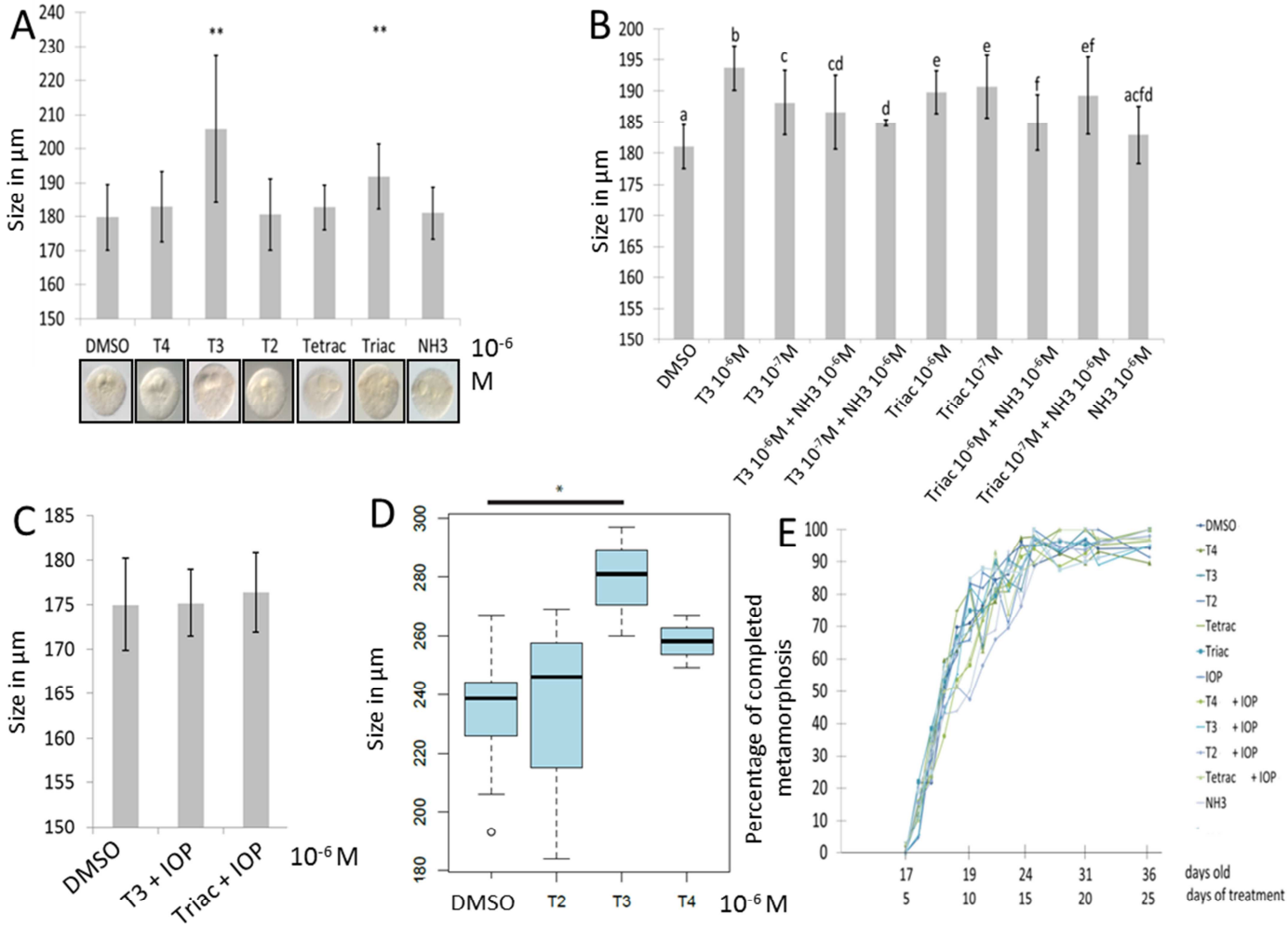

A
Size in µm
DMSO
T4
T3
T2
Tetrac
Triac
NH3
10-6 M
B
Size in µm
DMSO
T3 10-6M
T3 10-7M
T3 10-6M + NH3 10-6M
T3 10-7M + NH3 10-6M
Triac 10-6M
Triac 10-7M
Triac 10-6M + NH3 10-6M
Triac 10-7M + NH3 10-6M
NH3 10-6M
C
Size in µm
DMSO
T3 + IOP
Triac + IOP
10-6 M
D
Size in µm
DMSO
T2
T3
T4
10-6 M
E
Percentage of completed metamorphosis
DMSO
T4
T3
T2
Tetrac
Triac
IOP
T4 + IOP
T3 + IOP
T2 + IOP
Tetrac + IOP
NH3
days old
days of treatment
F
Relative gene expression
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
168 hpf
2 months
Immature
Male
Female
G
Relative gene expression
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
168 hpf
2 months
Immature
Male
Female

Figure 2. *Platynereis dumerilii* early growth is influenced by TH pharmacology. (A) Pharmacological treatment of *P. dumerilii* larvae with TH derivatives T4, T3, T2, Tetrac, Triac and the TR antagonist NH3. Treatments were performed at 10^{-6} M started at 5 hpf and stopped at 48 hpf. All chemicals are dissolved in DMSO. Worm size in µm is in the Y axis, the intersection of the X axis is at 150µm. Representative worm are shown in the lower panel. ** indicates significate differences of p-value < 0.01 compared to the DMSO condition. (B) Pharmacological treatment of *P. dumerilii* larvae with TH derivatives T3 and Triac at 10^{-6} or 10^{-7} M with or without NH3 at 10^{-6} M at 5 hpf and stopped at 48 hpf. All chemicals are dissolved in DMSO. Worm size in µm is in the Y axis, the intersection of the X axis is at 150µm. Letter a to f indicated statistical differences of p< 0.01. (C) Pharmacological treatment of *P. dumerilii* larvae with TH derivatives T3 and Triac with IOP. Treatments were performed at 10^{-6} M started at 5 hpf and stoped at 48 hpf. All chemicals are dissolved in DMSO. Worm size in µm is in the Y axis, the intersection of the X axis is at 150µm. (D) Pharmacological treatment of *P. dumerilii* larvae with TH derivatives T4, T3 and T2. Treatments were performed at 10^{-6} M started at 3 dpf and stopped at 5 dpf. Worm size in µm is in the Y axis, the intersection of the X axis is at 180µm. ** indicates significate differences of p-value < 0.05 compared to the DMSO condition. (E) Pharmacological treatment of *P. dumerilii* lworms with TH derivatives T4, T3, T2, Tertac, Triac and the antagonist NH3 with and without IOP. Treatments were performed at 10^{-6} M started at 12 dpf and stopped at 36 dpf. The percentage of worm having completed their cephalic metamorphosis is on the Y axis, worm age and treatment day are in the X axis. (F) Quantitative PCR following the expression of *tr* during the development of *P. dumerilii*. The relative expression of TR is on the Y axis ad worm stages on the X axis. Error bars are standard deviation of measurement performed in duplicate. The gene *rsp*, *hsp70* and *cdc5* were used are reference genes. (G) Quantitative PCR following the expression of one deiodinase during the development of *P. dumerilii*. The relative expression of TR is on the Y axis ad worm stages on the X axis. Error bars are standard deviation of measurement performed in duplicate. The gene *rsp*, *hsp70* and *cdc5* were used are reference genes.

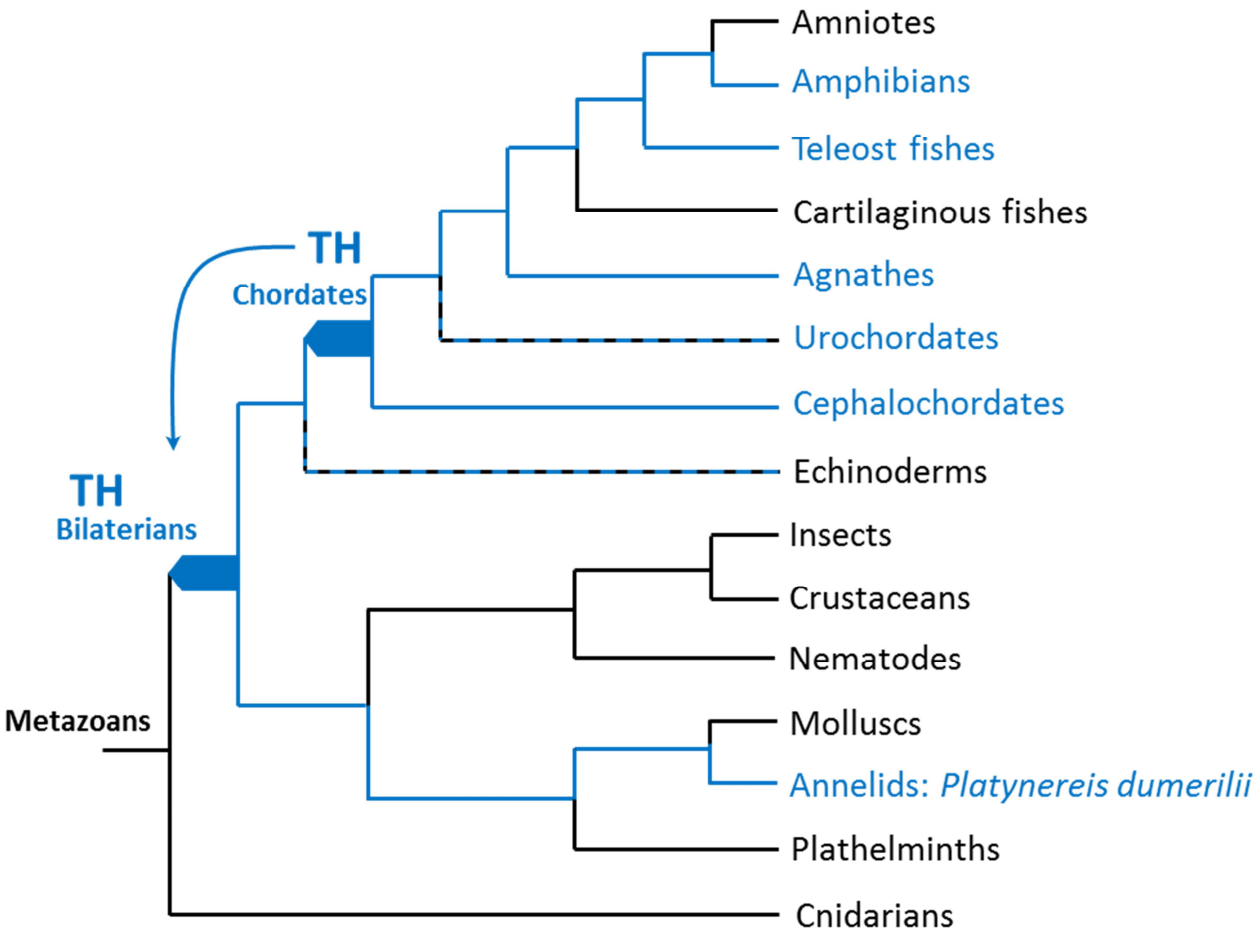


Figure 3. TH signaling is a synapomorphy of bilaterian and was certainly involve in development processes. Schematic tree of the main bilaterian taxa. Taxa undergoing a TH-controlled metamorphosis are highlighted in blue. We propose that it is the case for *P. dumerilii*. Taxa for which pharmacological evidence tends toward a TH controlled metamorphosis but still lacking molecular evidence are hatched in blue and black. We propose to push the origin of TH signaling back to the bilaterian origin as indicated by the blue arrow.

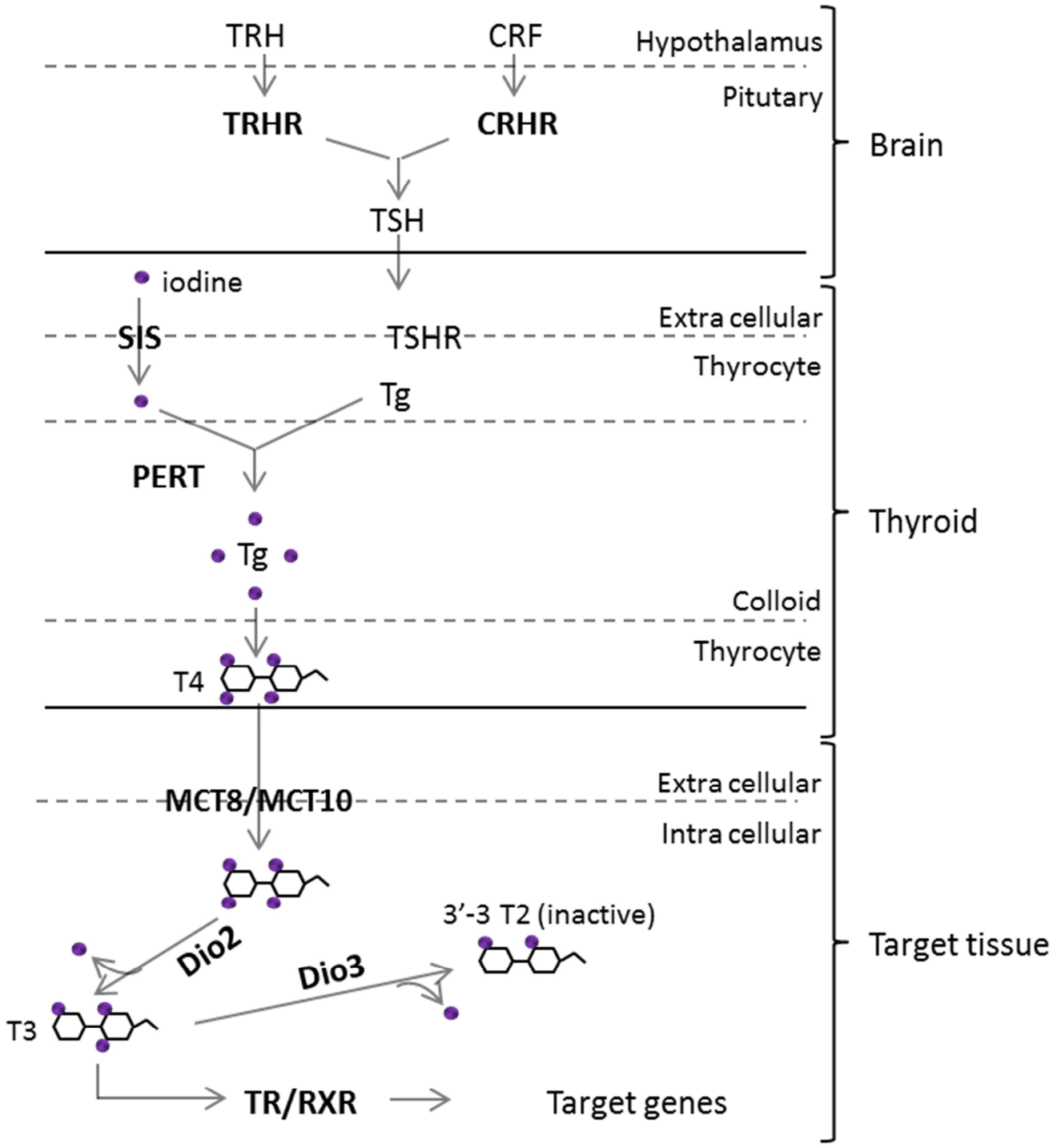


Figure S1. Orthologs of vertebrates TH signaling genes in *P. dumerilii.* Schematic view of the TH signaling in vertebrate adapted from (Paris, Brunet, *et al.*, 2008). Genes with identified orthologs in *P. dumerilii* are highlighted

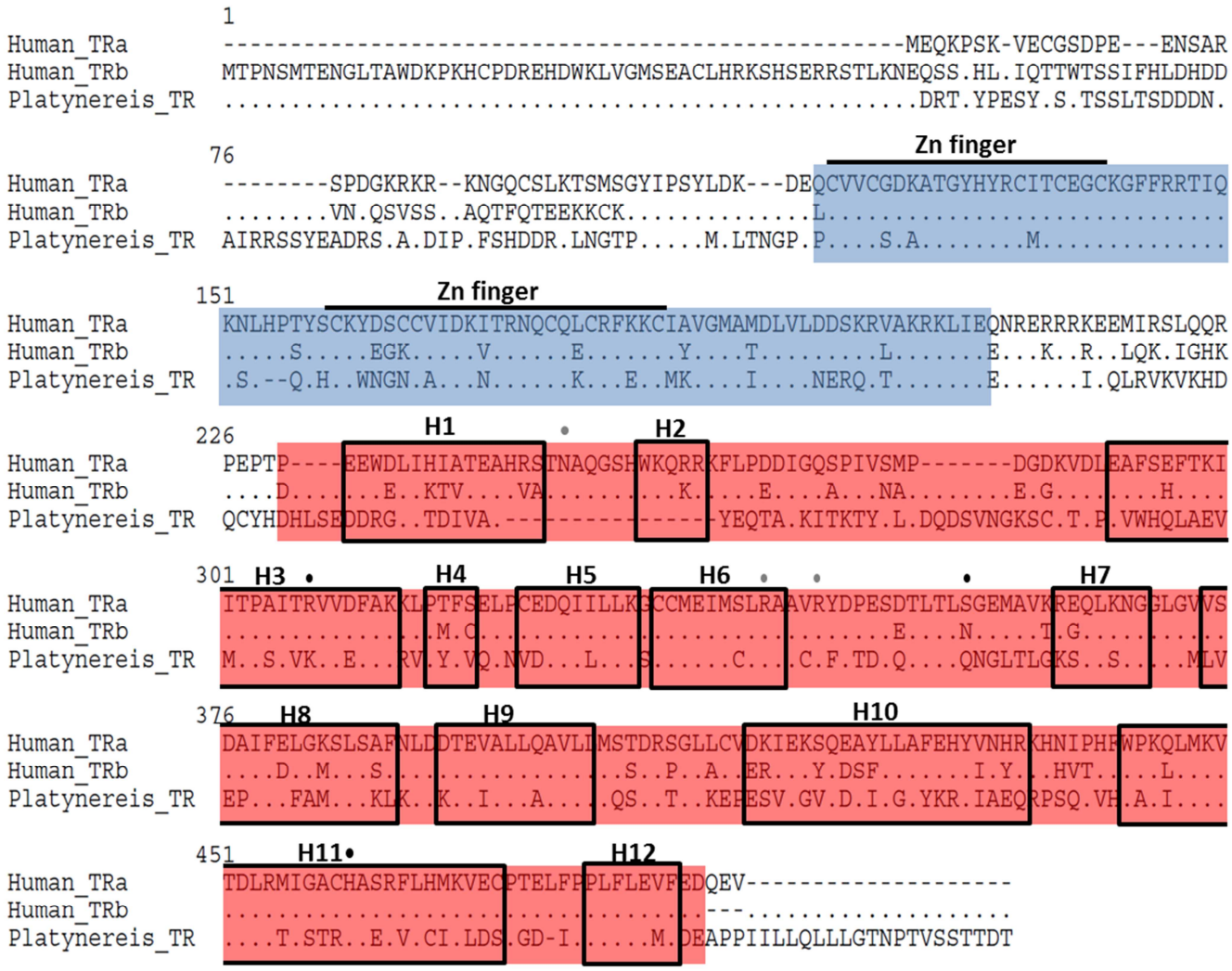
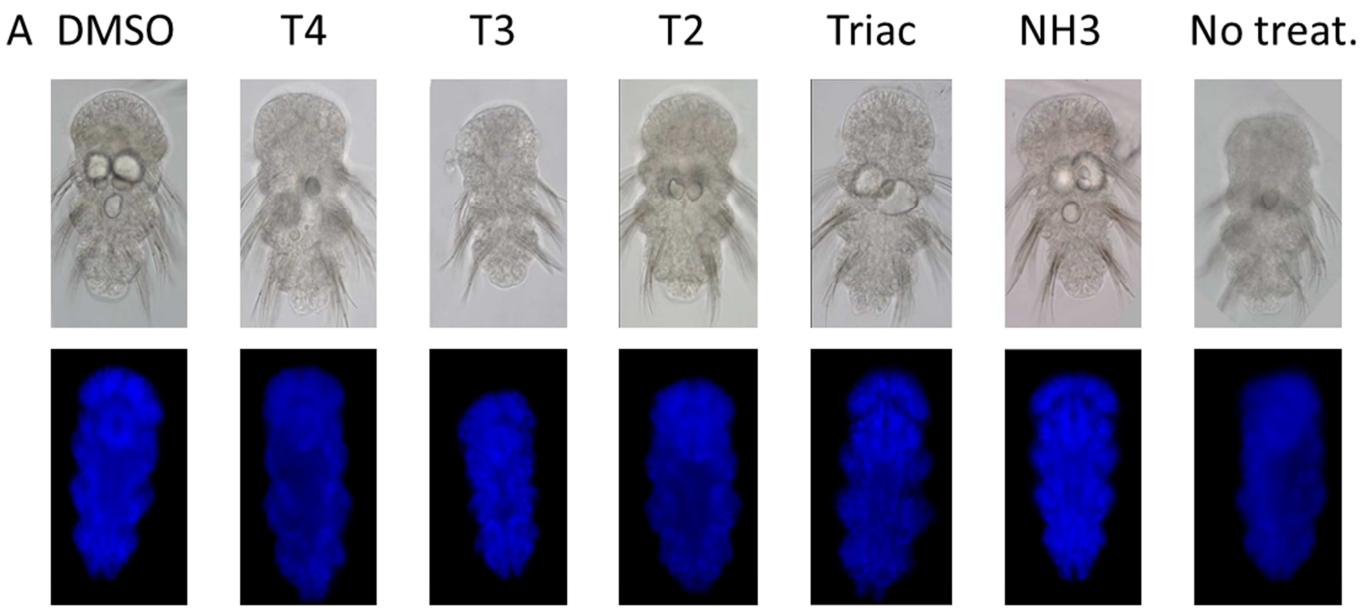
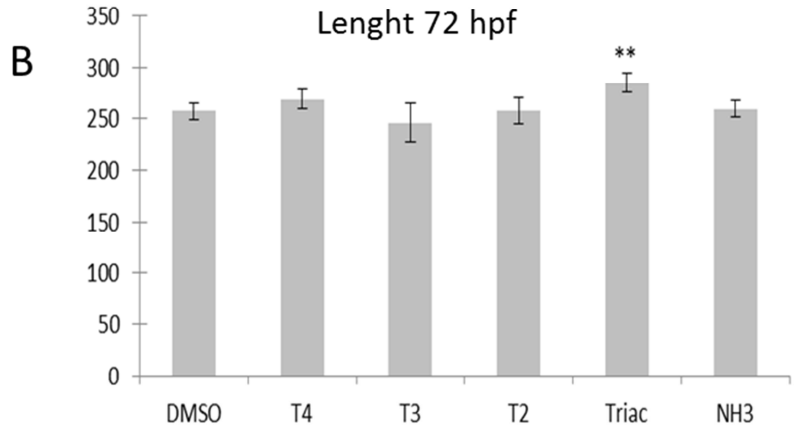


Figure S2. *P. dumerilii* TR exhibits similarities with and human TRs. Alignment of the protein sequences of P. dumerilii TR and human TRα and TRβ. Blue rectangle highlight the DBD, red rectangle highlight the LBD, zinc finger are indicated by black lines and helix of the DBD by boxes named H. Residues that form direct or indirect hydrogen bound with the ligand in the human TR pocket are respectively indicated with a black or grey dot.





Lenght	DMSO	T4	T3	T2	Triac	NH3
Mean	257,25	268,833333	246,222222	257,944444	284,8	259,571429
Sd	7,69171632	9,72426297	18,6151992	12,9814661	8,25860433	6,78380004
p-value		0,01312237	0,05556692	0,8579452	**1,1796E-06**	0,65601609

Figure S3. TH derivative treatment effects smoothen at 72 hpf. Pharmacological treatment of *P. dumerilii* larvae with TH derivatives T4, T3, T2, Triac and the TR antagonist NH3. reatments were performed at 10^{-6} M started at 5 hpf and stopped at 72 hpf. All chemicals are dissolved in DMSO. (A) Representative worm are shown in the upper panel. (B) Worm size in µm is in the Y axis, the intersection of the X axis is at 150µm. ** indicates significate differences of p-value < 0.01 compared to the DMSO condition. Length of the treated worms in µm are indicated.

A

48 hpf	DMSO	T4	T3	T2	Tetrac	Triac	NH3
Mean	179,810811	182,956522	205,684211	180,627907	182,787879	191,794872	181,0625
Sd	9,61144525	10,3343151	21,5665704	10,4972179	6,60850577	9,6086022	7,66979777
p-value		0,89363102	**7,3196E-08**	0,71918789	0,14035523	**6,7544E-07**	0,50604059

B

48 hpf	DMSO	T3 10^{-6} M	T3 10^{-7}M	T3 10^{-6} M NH3 10^{-6}M	T3 10^{-7} M NH3 10^{-6}M	Triac 10^{-6} M	Triac 10^{-7} M	Triac 10^{-6} M NH3 10^{-6}M	Triac 10^{-7}M NH3 10^{-6}M	T3 10^{-6} M
Mean	181,069	193,7143	188,15	186,625	184,875	189,8125	190,68421	184,933333	189,266667	182,9167
Sd	3,51457	3,58262	5,15318	5,9427827	0,5	3,4490337	5,0668052	4,431489056	6,16981904	4,581749
p-value		**8,61E-14**	**0,00146**	**0,0005734**	**0,0163889**	**4,154E-10**	0,5637766	**0,001814511**	0,46709528	0,169486

C

48 hpf	DMSO	T3	Triac
Mean	175	175,2	176,363636
Sd	5,18411034	3,765339	4,47823017
p-value		0,91622195	0,48061757

Table S1. TH derivative treatment effects at 48 hpf. (A) Length in µm of the *P. dumerili* larvae treated with TH derivatives T4, T3, T2, Triac and the TR antagonist NH3. Treatments were performed at 10^{-6} M started at 5 hpf and stopped at 48 hpf. p-value < 0,05 are highlighted in bold. (B) length in µm of the *P. dumerili* larvae treated with TH derivatives T3 and Triac at 10^{-6} or 10^{-7} M with or without NH3 at 10^{-6} M. Treatments started at 5 hpf and stopped at 48 hpf. p-value < 0,05 are highlighted in bold.

(C) length in µm of the *P. dumerili* larvae co-treated with TH derivatives T3 and Triac with IOP at 10^{-6} or 10^{-7} M. Treatments started at 5 hpf and stopped at 48 hpf.

Gene	Sens	Anti-sens	Comment
tr	CTCATCAACCAGCAGTCTGAC	GTTCCATTTGCAGTGGTACTG	partial cloning
tr	**NNNNGGATCC**ATGGATCGAACCCCATATCCAGAG	**NNNNGGATCC**TTAAGTATCGGTAGTCGAGGAGAC	full lenght cloning
tr	**NNNNCTCGAG**tcGAAGAAAACCGAGAGCGGCGAC	**NNNNGGATCC**TTAAGTATCGGTAGTCGAGGAGAC	LBD cloning
dio	TGGATAAATCGAAACTGAAACCAG	CATCCAAACGGTAGTCCC	partial cloning
tpo	GAGACTTGCGAGAACCAAGC	TCCACAGAAGGGTCGTAACC	partial cloning
tshr	CGACAGTCAAACCACCATTGAC	AACAGCCGACCACCAATAAGA	partial cloning
mct10	CCTGGTGCTTTGACTTGGATTC	GAAGGAACACTGCTGCATGAAC	partial cloning
crhr	TTGGATGTGGGGTTTCATTGG	GAAGCAGAACTTGCAATGGATG	partial cloning

Table S2. Sequences of the cloning primer used in this study.

Gene	Sens	Anti-sens	Comment
tr	GGTTTGTAGTGACGCTGCAAC	CCGTTCCATTTGCAGTGGTACT	designed for this study
dio	CAAATCAAATAGCAACTCTGTGGGAGG	TGTTCTCCTTAGGCATCTTAATGACTGG	designed for this study
hsp70	CGAACCAACAGCCGCTGC	CACATCGAAGGTTCCTCCTCC	designed for this study
rsp9	CGCCAGAGAGTTGCTGACT	ACTCCAATACGGACCAGACG	from Dray *et al.* 2010
cdc5	CCTATTGACATGGACGAAGATG	TTCCCTGTGTGTTCGCAAG	from Dray *et al.* 2010
α-tubulin	GCCAACCAGATGGTCAA	GCTTGGTCTTGATGGTG	from Altincicek and Vilcinskas 2007

Table S3. Sequences of the qPCR primer used in this study.

Specie	Protein	Accescion number	Database	Specie	Protein	Accescion number	Database
Acanthopagrus schlegelii	TRα	ABQ96861.1	NCBI	*Lytechinus variegatus*	TR	SPU_025239	Echinobase
Acanthopagrus schlegelii	TRβ	ABQ96862.1	NCBI	*Monodelphis domestica*	TRα	NP_001184135.1	NCBI
Ambystoma mexicanum	TRα	AY174871.1	NCBI	*Monodelphis domestica*	TRβ	XP_007505233.1	NCBI
Ambystoma mexicanum	TRβ	AY174872.1	NCBI	*Mus musculus*	RARα	NP_033050.2	NCBI
Anolis carolinensis	TRα	XP_008111558.1	NCBI	*Mus musculus*	TRα	NM_178060.3	NCBI
Anolis carolinensis	TRβ	XP_008117165.1	NCBI	*Mus musculus*	TRβ	NM_001113417.1	NCBI
Branchiostoma bleicheri	TR	ACR15148.1	NCBI	*Necturus maculosus*	TRα	Y16623.2	NCBI
Branchiostoma floridae	TR	EF672344.1	NCBI	*Necturus maculosus*	TRβ	AY168331.1	NCBI
Branchiostoma lanceolatum	TR	ABS11250.1	NCBI	*Oreochromis niloticus*	TRα-A	ENSONIT00000008144	Ensembl
Callorhinchus milii	TRα	103172030	KEGG	*Oreochromis niloticus*	TRα-B	ENSONIT00000022999	Ensembl
Callorhinchus milii	TRβ	103179946	KEGG	*Oreochromis niloticus*	TRβ	ENSONIT00000012974	Ensembl
Canis lupus familiaris	TRα	NP_001273791.1	NCBI	*Oryzias latipes*	TRα	AB114860	NCBI
Canis lupus familiaris	TRβ	XP_862690.2	NCBI	*Oryzias latipes*	TRβ	AB114861	NCBI
Capitella teleta	TR	Capca1|219252	JGI	*Paracentrotus lividus*	TR	none	personal communication
Conger myriaster	TRα-A	AB183396.1	NCBI	*Paralichthys olivaceus*	TRα-A	D16461.1	NCBI
Conger myriaster	TRα-B	AB183397.1	NCBI	*Paralichthys olivaceus*	TRα-B	D16462.1	NCBI
Conger myriaster	TRβ-A	AB183394.1	NCBI	*Paralichthys olivaceus*	TRβ	D45245.1	NCBI
Conger myriaster	TRβ-B	AB183395.1	NCBI	*Patiria miniata*	TR	HP130651.1	NCBI
Crassostrea gigas	TR	EKC30867.1	NCBI	*Petromyzon marinus*	TR1	DQ320317.1	NCBI
Danio rerio	TRα-A	NM_131396.1	NCBI	*Petromyzon marinus*	TR2	DQ320318.1	NCBI
Danio rerio	TRα-B	XM_001920978	NCBI	*Platynereis dumerilii*	TR	in this study	in this study
Danio rerio	TRβ	NM_131340.1	NCBI	*Rattus norvegivus*	TRα	NP_001017960.1	NCBI
Epinephelus coioides	TRα-A	ABP62960.1	NCBI	*Rattus norvegivus*	TRβ	NM_012672	NCBI
Epinephelus coioides	TRα-B	ABP62961.1	NCBI	*Saccoglosus kowaleskii*	TR	GU076139.1	NCBI
Epinephelus coioides	TRβ	ABP62962.1	NCBI	*Salmo salar*	TRα	NM_001123628	NCBI
Eublepharis macularius	TRα	AB204861	NCBI	*Salmo salar*	TRβ	NM_001123700	NCBI
Eublepharis macularius	TRβ	AB204862	NCBI	*Strongylocentrotus purpuratus*	TR	XM_784395	NCBI
Gallus gallus	TRα	NM_205313.1	NCBI	*Sus scrofa*	TRα	NM_214190	NCBI
Gallus gallus	TRβ	NM_205447.1	NCBI	*Sus scrofa*	TRβ	F1RS47_PIG	Ensembl
Homo sapiens	TRα	NM_199334.3	NCBI	*Xenopus laevis*	TRα	NP_001081595	NCBI
Homo sapiens	TRβ	NM_000461.4	NCBI	*Xenopus laevis*	TRβ	NP_001090182	NCBI
Homo sapiens	RARα	NP_001138773.1	NCBI	*Xenopus tropicalis*	TRα	NM_001045796.1	NCBI
Lottia gigantea	TR	Lotgi1|171663	JGI	*Xenopus tropicalis*	TRβ	NM_001045805.1	NCBI

Table S4. Sequences used in this study to build the TR phylogenetic tree

Probe	Sequence
DR0	GCGATTTG**AGGTCAAGGTCA**CACAGTTATA
DR1	GCGATTTG**AGGTCA**G**AGGTC**ACACAGTTAT
DR2	GCGATTTG**AGGTCA**AG**AGGTCA**ACAGTTA
DR3	GCGATTTG**AGGTCA**CAG**AGGTCA**CACAGTT
DR4	CGATTTG**AGGTCA**CAGG**AGGTCA**CACAGTT
DR5	CGATTTG**AGGTCA**CCAGG**AGGTCA**CACAGT
DR8	ATTTG**AGGTCA**AGGTCAAG**AGGTCA**CACAG
Non-specific	AGCCTTCGAGCCAAAATGTTCCAAAGACTGT

Table S5. Sequences of the ^{32}P labelled and cold probes used for the EMSA

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2.3. Thyroglobulin: novel molecular design conserved in all vertebrates

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Abbreviated **title:** Thyroglobulin conservation in vertebrates

Key words: Thyroid Hormones, Thyroglobulin, Metamorphosis, Zebrafish, Xenopus

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Abstract

Thyroid hormones (THs) are multi-faceted hormones in vertebrates, playing a role in the control of post-embryonic life stages transition, energy metabolism as well as seasonality. Despite this importance the production of TH from a large protein precursor, thyroglobulin, has been studied only in vertebrates and there is therefore a strong bias in our knowledge of how Th are produced in other organisms. In this paper we searched for Tg genes in genomic data available in both vertebrates and non-vertebrate species. We did not found any orthologous of Tg among non-vertebrate species, strongly suggesting that Tg is a vertebrate-specific protein. In contrast we found Tg in all vertebrates, since we found a clear Tg orthologue in the sea lamprey *Petromyzon marinus*. We observed that the different domains of the vertebrate Tgs are conserved in vertebrates. Interestingly Although the overall amino acid sequences are quite divergent between human, *Xenopus*, zebrafish and lamprey, key residues such as the hormonogenic tyrosines and the disulfide bond forming cysteins are well conserved. This is striking as these key conserved amino acids are exceptions; indeed, despite the overall structural similarity the 4 vertebrate Tgs sequences we studied are strongly divergent The biochemical characterization of experimentally cloned Tg sequences in zebrafish and xenopus extend our knowledge of Tg function outside vertebrates. This characterization coupled to the overall structural similarities of Tg in all vertebrates, and the absence of recognizable Tg outside vertebrate indicate that Tg is a novel design elaborated once at the base of vertebrates and kept very similar, despite sequence divergence, in most vertebrates.

Introduction

Among the many signalling molecules that regulate human physiology, thyroid hormones (TH) are one of the most puzzling. Indeed these hormones are known to control different processes in different species (Tata, 2013). In many amphibians as well as in teleost fish and some invertebrate chordates, THs trigger metamorphosis, a spectacular life-history transition (Laudet, 2011). More recently THs have been implicated in the control of seasonality, a process during which animals adapt their physiology, reproductive function and behavior to the annual change of photoperiod (Dardente *et al.*, 2014). In mammals, THs are involved in crucial physiological functions including oxidative metabolism, heart rate, and thermogenesis (Gong *et al.*, 1997; Mullur *et al.*, 2014; Williams, 2014). This is particularly important for human health since ~10% of the population of developed countries is at risk of developing a thyroid related disease during their lifespan(Hollowell *et al.*, 2002). How such different functions can be reconciled in a common framework is still an open question (Holzer & Laudet, 2015).

In addition to these disparate functions, THs are also very unusual when considering their mode of production. First, the reason for the requirement of iodine in a compound with hormonal activity is still a mystery (Miller & Heyland, 2013). Second, TH are synthesized from a large dimeric protein, thyroglobulin (Tg) which contains 2749 amino acid in human and is specifically produced in the thyrocyte, the main cell type of the thyroid gland (Malthiéry & Lissitzky, 1987; van de Graaf *et al.*, 2001).

Given the importance of TH in controlling major life history transitions in many species, it is striking to realize that the only cloned and experimentally characterized Tgs come from mammals (Table S1A; Mercken *et al.*, 1985; Malthiéry & Lissitzky, 1987; Kim *et al.*, 2000; Lee *et al.*, 2007) although many aspects of physiology (*e.g*. regulation of body temperature) and development in the main vertebrate clades are different from those of mammals. Outside of mammals, all other available Tg sequences are only predictions from genome assembly or EST data (Table S1B), and are known only from Gnathostomes. As a consequence, how the TH synthesis system emerged during evolution remains unknown. There have been some descriptions of possible Tg orthologs in invertebrate chordates, but those assignments remain controversial (Belkadi *et al.*, 2012) . It is particularly notable that a complete Tg has

not been characterized neither in *Xenopus* nor in zebrafish, two major model systems used to decipher the role of TH in physiology and development.

From the data accumulated in mammals it appears that Tg undergoes a complex trafficking between the thyrocytes and the lumen of the gland. Newly-synthesized Tg is glycosylated, folds to include many internal disulfide bonds, and assembles into a large 660 kDa homodimer in the thyrocyte, before secretion to the lumen of the thyroid gland (A. J. Van Herle *et al.*, 1979; A. Van Herle *et al.*, 1979; Kim & Arvan, 1991; van de Graaf *et al.*, 2001). In the lumen, near the thyrocyte surface, tyrosines of the Tg are iodinated by thyroid peroxidase and specific iodotyrosine pairs are coupled together by the same enzyme to form TH within the Tg protein (Lamas *et al.*, 1989; Ris-Stalpers & Bikker, 2010). This process is extraordinarily costly since it requires two 2749 amino-acid proteins to produce one or two TH molecules, each formed from only two modified tyrosines.

The iodinated Tg molecules bearing pre-formed TH stay in the lumen. Upon stimulation of hormone release there is an uptake of iodinated Tg from the colloid by the thyrocytes. This is followed by Tg delivery to lysosomes and extensive proteolytic cleavage of the protein, resulting in the liberation of TH molecules that are then released into the bloodstream (Brix *et al.*, 1996; Rubio & Medeiros-Neto, 2009). It is known that most TH formed in the thyroid gland is thyroxine (T4, ~80%) which contains 4 iodine atoms from the coupling of two diiodotyrosine residues. T4 itself is poorly active at the receptor level (Chopra, 1996; Flamant *et al.*, 2006). A smaller fraction of TH produced within the thyroid gland is comprised of T3 (triiodothyronine) which contains only 3 iodine atoms and which is the active form of the hormone. T4 released from the thyroid gland can also be converted to T3 in tissues that express thyroxine deiodinases, which help to tightly control the amount of available active hormone within each target organ(Bianco & Kim, 2006) .

Thyroglobulin is a multi-domain protein that harbors different modules, from N-terminal to C-terminal (Figure 1): a signal peptide, four Tg type 1 repeats (Tg1), a linker region, six more Tg1 repeats, a hinge region, three Tg type 2 repeats (Tg2), one Tg1 repeat, five type 3 repeats (Tg3) and one large CholineEsterase-Like (ChEL) domain. The signal peptide is necessary to address the protein into the endoplasmic reticulum of the thyrocyte. The Tg repeats are notable features of the protein structure, containing many cysteine residues that form numerous intra-domain disulfide bonds required for the correct conformation of the protein (Lee & Arvan, 2011). The ChEL domain is implicated in dimerization, conformational

maturation, and the trafficking of newly-synthesized Tg to the colloid (Park & Arvan, 2004; Lee & Arvan, 2011).

The various domains present in Tg are more ancient than Tg itself (Krejci *et al.*, 1991; Novinec *et al.*, 2006; Belkadi *et al.*, 2012). By studying the evolution of the Tg1 repeats, Novinec *et al.*, 2006 found that these repeats are indeed present in all bilateral taxa whereas there is no firm evidence of Tg outside Gnathostomes. In addition, the ChEL domain is present in other proteins with very different activities such as acetylcholinesterase itself but also other esterases, neuroligins, etc…(Krejci *et al.*, 1991).

Along the human Tg sequence, some residues are particularly important for the protein function. The tyrosine Y5 and Y130 are respectively known as acceptor and donor for the most important hormonogenic site, producing nearly half of the T4 in Tg (Dunn *et al.*, 1989; Palumbo *et al.*, 1990). Several other hormonogenic tyrosines have been identified (*e.g.*, Y847, Y1291, Y1448, Y2554; Ohmiya *et al.*, 1990) with Y2747 being the most important T3 formation site. Aside from tyrosines, cysteines are important for Tg structure by their engagement in disulfide bonds especially in the repeat domains. Indeed the spacing between cysteine residues allows the precise identification the distinct Tg repeats (Malthiéry & Lissitzky, 1987).

As discussed above it is unclear how much the main aspects of TH synthesis are conserved among the vertebrates. Moreover, outside from vertebrate no functionally relevant domain or structure reminiscent of Tg has been identified and the TH synthesis mechanisms remain unknown. Identifying functionally relevant regions or residues from those that are less important may may help the search for distant Tg orthologues outside of vertebrates if those exists. For these reasons, we have cloned and characterized Tg in *Xenopus* and zebrafish, two major non-mammalian experimental models. To complete our view of vertebrate Tg evolution, we have also searched genomic data accumulated in lamprey (*Petromyzon marinus*) as well as in genome sequences outside vertebrates. Herein we report extensive conservation of Tg organization and structure across the whole vertebrate clade, strongly suggesting that the mechanism of Tg synthesis is conserved throughout the vertebrates, whereas no clear sign of Tg presence outside of vertebrates has been detected. Thus the evidence suggests that the "Tg design" was set up at the base of vertebrates and has remained conserved since that time.

Material and Method

Cloning of the *Xenopus* and zebrafish Tg.

RNA from the head of *Xenopus* (*Xenopus tropicalis*) and total RNA from zebrafish (*Danio rerio*) were extracted with Macherey-Nagel RNA extraction kit following the manufacturer instructions. They were and used for retro-transcription using the Super-Script III (Invitrogen) following the manufacturer's protocol. The cloning of both genes has been performed in two steps: (*i*) Primer were designed following the genes's prediction available in Genebank (XM_689200.4 and XM_002939515.1 for zebrafish and xenopus respectively) in order to obtain 1000 pb long overlapping amplicon. PCR were performed with Platinium Taq (Invitrogen) following the manufacturer protocol. Amplicons were cloned in the plasmid PCRII and sequenced in both sense using the M13 and M13R primers by GATC. For both genes, the full length sequences were reconstructed from these overlapping partial sequences. (*ii*) Primers were designed following the sequencing result in order to obtain about 2000 pb long amplicon with 15 overlapping nucleotides at each ends of the primers. PCR were performed with the Phusion High-Fidelity polymerase (Thermo Scientific) following the manufacturer recommendations. Primer used for these various steps are listed in Supplementary Table 2. For each genes 4 amplicons were obtained, assembled and cloned in pSG5 plasmid between EcoRI (5') and BglII (3') by In-Fusion cloning (Thermo Scientific) following the manufacturer recommendations.

Cloning of the 5'-end of *Xenopus* Tg

We failed to clone the 5'-end of the Xenopus Tg with the method described above and we had to use another strategy to find the missing 5'-end encoding the signal peptide. We used the Teloprime cDNA amplification kit from Lexogen following the manufacturer recommendations to clone the missing 5'-end. To do so, we designed two reverse primers in the 5' part, to be use with two forward primers of the kit. (Table S2)We performed a PCR and a nested PCR with those primers. The amplicon mix resulting from these PCRs was sequenced with an Ion Torrent PGM sequencer from Life technologies. We compare the sequencing result with the Xenopus genome available on Ensembl to identify the 5'-end of

the Xenopus Tg (underlined in Figure S1). This region was then chemically synthetized and incorporated in the previous incomplete clone to generate a complete clone encoding the full-length Xenopus Tg.

Phylogenetic analysis

Phylogenetic trees were built with the available amino acid sequence of human, dog, cow, rat, mouse (Table S1A), *Xenopus* and zebrafish (from the present paper). Predicted sequences were also used for phylogeny (Table S1B).

The Tg amino acid sequences were aligned using MUSCLE (Edgar, 2004). Trees were generated using the maximum likelihood method under a JTT substitution matrix plus a eight category gamma rate correction (α estimated) with the proportion of invariant sites estimated and 1000 bootstrap replicates. Both alignment and tree calculation were performed using the Seaview v4.5 software (Gouy *et al.*, 2010).

Search for Tg sequences in non-vertebrate genomes

To investigate Tg sequences in non-vertebrate genomes, we searched for Tg1, Tg2 or Tg3 repeats using TBLASTN and TBLASTX(Altschul *et al.*, 1997) against some available sequences of tunicate, cephalochordate, echinoderm, hemichordate, mollusk, annelid, cnidarian and xenocoelomorph (Table S3) To assess if the retrieved sequences were orthologs of thyroglobulin Tg-domains and not of Tg domains found in other unrelated proteins such as Nidogen, SMOC or TACSTD we built the phylogeny of all retrieved repeats (Table S4).

A prediction for Tg (ENSPMAG00000001187) was recovered from the lamprey (*Petromyzon marinus*) genome assembly avalaible on ENSEMBL (Smith *et al.*, 2013). The sequence were checked by BLAST, the scaffold GL476337 containing the gene prediction was run with AUGUSTUS (Sommerfeld *et al.* 2009) in order to increase the protein prediction length on both N-terminal and C-terminal ends.

In situ hybridization in zebrafish and *Xenopus*

Two different probes were tested corresponding to respectively the 5' (probe 1; 1709 bp long; position 42-602 in the amino acid sequence, see Figure S1) and the middle (probe 2; 1994 bp; position 1202-1862 in the amino acid sequence) of the Tg coding sequence. Probes were synthetized using DIG linked UTP and T3 polymerase (Roche) following the manufacturer instructions. Probes were treated with DNAse I (Roche) and purified on column (ENZA) following the manufacturer's instructions.

1-phenyl-2-thiourea (PTU) at a final concentration of 0.2 mM was added to the embryos to prevent the development of endogenous pigments. Zebrafish embryos harvested at the desired development point, were fixed in PFA4% overnight at 4°C and dehydrated to be stored in methanol. For the *in situ* hybridization, larvae were rehydrated, pre-treated with proteinase K 20 min for 30 hpf to 60 min for 120 hpf and more and then re-fixed in PFA 4°C 20 min. A pre-hybridization step in the hybridization buffer was carried out 4 hours at 70°C, and hybridization was performed overnight at 70°C. Excess probe was washed in successive bath of hybridization/SSC buffer. Embryos were pre-incubated in the antibody buffer for 4 hours at room temperature and incubated with an anti-DIG antibody at 4°C overnight. Excess of antibody was washed in PBT medium. Probe revelation was performed using BM purple reagent (Roche) following the manufacturer instruction at room temperature. Less than 8 hours of incubation was necessary to obtain a signal. Staining reaction was stopped with a fixation in PFA 4% overnight at 4°C and the embryo were cleared in glycerol batch for observation and storage.In situ hybridization were performed on *Xenopus tropicalis* tadpole as previously described (Fini *et al.* 2010).

Biochemical characterization of zebrafish and *Xenopus* Tg

Results

Cloning of *Xenopus* and zebrafish full-length Tg

Using RT-PCR with primers designed from the partial predicted sequences available in complete genome data, we cloned full-length Tgs from *Xenopus* and zebrafish. In zebrafish, we were able to isolate a full-length clone that encodes a 2733 amino acid protein harboring a very similar organization to the human Tg protein (Figure 1).

Using the same strategy we isolated a *Xenopus* clone that is incomplete at the 5'-end. This region (starting from position 4: EYQL...) corresponds to an exon whose position is conserved. Upstream to this exon the contig in question contains ~9 kbp of sequence with nothing reminiscent of the sequence corresponding to the signal peptide. Because the presence of such a peptide is mandatory for the function of the protein we decided to complete the clone in 5'. We designed two reverse primers following our partial cloning to be used with the Teloprime cDNA amplification kit. The obtained amplicon was then sequenced on a PGM Ion-Torrent. In the sequence data analyzed we found a sequence corresponding to this missing part of the Tg (about half out of 600 000 reads). Using this sequence we constructed a full-length *Xenopus* Tg clone encoding a 2780 amino acid protein that could be used, together with the zebrafish one, for biochemical characterization.

A Tg sequence in lamprey....

Early biochemical evidence suggests the existence in lamprey and amphioxus of a large molecular size iodinated protein present in the endostyle and/or thyroid gland (Suzuki *et al.*, 1975; Wright *et al.*, 1980, Monaco *et al.*, 1978, 1981). The corresponding clones were never isolated and the amphioxus genome has been extensively searched for a Tg ortholog without finding anything (Paris et al., 2008). We therefore searched for Tg orthologs in basal vertebrates (chondrichtyans and agnathans for which several genomes are available), as well as in the most recent versions of amphioxus, urochordate and echinoderm genomes.

We found a clear candidate for lamprey Tg. The retrieved sequence encodes a partial 2605 amino acid protein that contains the N-terminal part of Tg with the various Tg repeats as well as the C-terminal ChEL domain. However, it misses a part of the ChEL domain that is not present in the genome data (Figure 1). This explains the gap present between amino

acids 2453 and 2632 in the lamprey protein (Figure 1 Figure S1. Note that, for clarity, all amino acid positions cited in this paper use the human numbering system).

… but not outside vertebrates

Up to now Tg is known only in Gnathostomes with no clear Tg sequence available outside this clade. Whereas some authors have proposed Tg-like proteins in amphioxus and sea urchin, these sequences were found to contain only several clustered Tg1 repeats (Novinec 2006, Belkadi *et al.* 2012). As many proteins contain Tg1 repeats and in a lesser extend Tg2 repeats, the identification of those candidates as Tg orthologues appears dubious. To check this, we built a phylogeny with all these Tg 1 repeats. (Figure S2 A) None of the Tg1 sequence we tested, including some not previously analyzed in the literature: *Capitella teleta* and *Crassostrea gigas*), cluster with any the Tg1 repeats of Tg. This indicates those proteins harbouring Tg1 repeat are not orthologs of vertebrate Tg.

In *Strongylocentrotus purpuratus* and tunicates species, we retrieved several Tg2-like repeat identified in this species as EGF GCC2-GCC3 domains (according to NCBI identification). Again, these Tg2 repeat retrieved in non-vertebrate species do not cluster with Tg in a phylogenetic tree (Figure S2 B). Therefore, we conclude that these proteins are not orthologus of the vertebrate Tg. We did not retrieve any Tg3-like sequence outside of vertebrates. Together these results indicate that there is no clear Tg orthologs outside vertebrates metazoans.

Structural conservation coupled with sequence divergence of vertebrate Tgs

Figure 1 compares the organization of Tg in human, *Xenopus*, zebrafish and lamprey. It clearly shows that in all of these species, Tg contains all of the domains found in mammalian Tg. Indeed, the Tg of each of these species harbor eleven Tg1, three Tg2 and five Tg3 domains. Specifically, in contrast to previous reports, the seventh and ninth Tg1 repeats are indeed present in zebrafish Tg as well as those from other fishes (Figure S2). In all proteins, the Tg3 domains can be sub-characterized as type 3a and type 3b (called Tg3a and Tg3b, respectively). Moreover, *Xenopus*, zebrafish and lamprey Tgs exhibit a linker and a hinge region in the middle of the protein and a ChEL domain at the C-terminal end. Those regions

were identified as similar and of the same size as the mammalian one, due to their amino acid sequence and particularly the conserved cysteines. Taken together, these data indicate a strong conservation of the structural organization of the Tg protein.

This overall structural conservation is not reflected in terms of sequence conservation since Tg proteins are quite divergent in terms of overall amino acid conservation. Indeed the overall sequence conservation between human Tg and the Tg of other species are as follows: *Xenopus* 48%, zebrafish 43% and lamprey 31% (note than the lamprey value may be marginally affected by the lack of a part of the ChEL domain). Thus, the Tg proteins are actually quite divergent.

The prediction of the secondary structures of the four species reveals that the proteins are structured in α-helixes with some β-sheets (Figure S3). Interestingly, the structuration is in overall conserved between human, *Xenopus*, zebrafish and lamprey with the α-helixes located at the end of the Tg1 repeats and the β-sheets at the beginning of them. The linker domain is also well structured with 30% of α-helixes and 10% of β-sheets. An amphipathic helix is also predicted at the end of the linker with five negatively charges amino-acid on one side and five hydrophobic ones on the other. This helix could have a role in the interaction of Tg with other partners. Comparison of the Tg sequence with crystalized proteins on PDB identified the Tg reapet pattern CWCVD in the proteins P41ICF (accession number 1L3H) and ILGF4 (accession number 2DSR). Tg repeat form disulfide bonds in those proteins indicating that, despite having no crystal structure of Tg, the Tg repeats of the Tg are likely to form disulfide bounds as in those two proteins., indicating that the Tg1 repeat could be the backbone for the proper folding of the Tg.

Striking conservation of key residues highlight the functional conservation of Tgs.

The general sequence divergence of the various Tg proteins renders even more striking the strong conservation of key residues known to be important for Tg function. Those key amino acids include in particular the hormonogenic tyrosines (Dunn *et al.*, 1989; Lamas *et al.*, 1989). The two tyrosines mainly involved in TH synthesis in mammals, Y5 and Y130 (human numbering) are strictly conserved in *Xenopus*, zebrafish and lamprey, highlighting their functional importance. Of the other hormonogenic residues, Y2747 (primary site for T3 formation in mammals) is also strictly conserved in the four species. Y2554 is conserved in

Xenopus and zebrafish but falls into a gap in the predicted lamprey sequence. Y1291 which is considered having a minor role in TH synthesis is conserved in zebrafish and lamprey but not in the *Xenopus*. Other hormonogenic tyrosines as Y847, Y1448 that have been described with minor activities in human Tg are not conserved (Figure S1). Therefore, in summary, the most potent hormonogenic tyrosines: Y5, Y130 and Y2747 are all conserved in human, *Xenopus*, zebrafish and lamprey.

Supplementary Figure 1 shows the complete sequence alignment of the 4 Tgs that highlight these key amino acids. Interestingly, the cysteines that are instrumental for correct folding of the protein are also strikingly conserved (Veneziani *et al.*, 1999). Indeed 90% of the cysteines present in the molecule are found at the same positions between human, *Xenopus*, zebrafish and lamprey, whereas only 14% of the amino acids overall are conserved between the four studied proteins. In *Xenopus*, there are two missing cysteines equivalent to the human positions 1023 and 1030 (repeat Tg1-8), which from a disulfide pair at the orthologous position in human Tg (Figure S1), suggesting coevolution of these two positions in *Xenopus*. In zebrafish there are also two missing cysteines (at position 343 and 1262) but despite these minor differences, the organization of the disulfide bond network within Tg proteins appears tightly conserved amongst the Gnathostomes.

In Lamprey, there are missing cysteines at position 114 and 120 (repeat Tg1-2), 1146 and 1166 (repeat Tg1-9) and 1558 and 1569 (Tg1-11 repeat). Interestingly, the orthologs of these cysteines are thought to be disulfide partners in human Tg. (Figure S1). Additionally, lamprey appears to be missing cysteine, 388 (linker), and 589 (repeat Tg1-5), the implication of which has not yet been tested experimentally, but the predicted secondary structure does not seem affected.

Finally, it is worth mentioning that, in contrast to other ChEL proteins, the catalytic site of the acetylcholine esterase domain is not conserved in the Tgs. This suggests that the loss of the catalytic affinity in this domain is ancient.

Phylogenetic analysis of Tg sequences

Amino acid sequences of Tg were used to build a ML tree using the experimental plus the predicted sequences (Figure 2). We however decided not to include the Tg sequence from

Latimeria in our analysis since this sequence appears quite fragmented and incomplete. The tree exhibits the expected topology with the phylogenetic relationships between the major vertebrate clades respected (Shen *et al.*, 2011). Most of the node exhibit a strong statistical support (>0.900 Figure 2) indicating an overall strong support of the topology.

Interestingly, we noticed in this phylogeny a very long branch supporting the group of percomorpha. This clearly indicates an acceleration of Tg evolution among this group. This acceleration is clearly visible but in a conserved context since, once again, the cysteins and the modular structure of Tg are conserved within percomorpha indicating that the overall features and function of the Tg should be conserved in this species. This acceleration might be linked to the fast radiation of percomorpha as rapid gene evolution has already been described is this group (Cortesi *et al.*, 2015).

Given the modular structure of Tg, we also calculated trees with each of the various domains (namely Tg1 only, Tg2, Tg3, Linker, Hinge and ChEL) isolated. Tg2 repeats which , appear more sensitive to individual substitution than other Tg domains given their short size (49 amino acids. However, overall, the trees calculated from individual domains exhibit the same topology as that seen in the full length tree, indicating strong structural conservation of the entire protein (Figure S4).

Expression of Tgs

We next studied the expression of Tg during embryogenesis in both zebrafish and xenopus. This was prompted by the recent description of extrathyroidal expression of Tg variant observed in mouse (Wu *et al.*, 2009). Because the Tg gene encodes a very large mRNA, we checked the expression pattern observed with two different probes corresponding to different Tg regions.

Expression of *tg* in zebrafish was studied by whole mount *in situ* hybridization at 4 different stages of development (Figure 3A). Two probes were designed, one at the 5' of the transcript and the other in the middle of the transcript. At 24 hpf, no expression was observed. At 48 hpf, *tg* is expressed in one localized spot in the pharyngeal region of the fish. At 120 hpf, the expression pattern increase in size and adopt an elongated shape, always in

the pharyngeal region. This corresponds to the organization of the teleost thyroid gland which, in contrast to the well-defined gland found in mammals, is scattered in several nodules to form a chain-like pattern along the pharyngeal region. The expression pattern was identical at 144 hpf and the same pattern was observed with the two probes, ruling out major extrathyroidal expression of an alternative transcript.

In the *Xenopus* tadpole, as in zebrafish, *tg* is expressed only in thyrocytes of the thyroid gland (Figure 3B). *In situ* hybridization on cross section revealed expression at the stage NF57 and NF59. Although ISH is not a quantitative method, the difference in labelling tends to indicate that the thyroid is more active at NF59 than NF57, consistent with increased TH levels at NF59 (Leloup & Buscaglia, 1977).

Biochemical characterization of *Xenopus* and zebrafish Tgs

The cDNAs encoding mouse, *Xenopus*, and zebrafish Tg were subcloned into eukaryotic expression vectors and transfected into 293T cells. Beginning after 24 h, cell culture medium was collected for one day and the cells were lysed, and both were analyzed by SDS-PAGE and Western blotting with a polyclonal antibody against rat Tg (Figure 4). As expected, there was very strong immunoreactivity of mouse Tg that was secreted efficiently from cells (C) to medium (M). Both *Xenopus* Tg and zebrafish Tg were also immunoreactive with polyclonal anti-rat Tg, but the signal strength was much weaker than for mouse Tg (Figure 4). However, whereas zebrafish Tg was secreted to the medium, *Xenopus* Tg was detected only within cell lysates (arrows, Figure 4). Because *Xenopus* Tg is not secreted and it lacks two cysteines that form the second disulfide bond in repeat 1-8 of mammalian Tg, we mutagenized these cysteine residues within mouse Tg. The double-cysteine mutant mouse Tg was well expressed and secreted (Figure 5); indeed, even single cysteine mutants preventing formation of the second Tg1-8 disulfide bond were secreted (not shown). We conclude that the second Tg1-8 disulfide bond is not required for Tg secretion and cannot explain the secretion defect of our *Xenopus* Tg cDNA.

To eliminate concerns about problems with immunoreactivity of *Xenopus* Tg with antisera against rat Tg, we introduced an XhoI restriction site at the 3' end of the coding sequence and at this site introduced an in-frame myc epitope tag into our Tg cDNAs. *Xenopus* Tg-myc was well expressed; however, as with the untagged protein, it was not secreted from cells to

medium (Figure 6A). Moreover, as judged by sensitivity to endoglycosidase H or PNGase F, mouse Tg-myc acquired asparagine-linked oligosaccharides indicating delivery of the Tg polypeptide into the lumen of the endoplasmic reticulum (Figure 6B, *left*), whereas *Xenopus* Tg-myc migrated as an unglycosylated protein and was not further digested by endoglycosidase H or PNGase F (Figure 6B, *right*). These data strongly suggested that the protein encoded by our *Xenopus* Tg cDNA was not delivered into the lumen of the endoplasmic reticulum. However, such a result seems incompatible with the presence of highly conserved cysteine pairs that appear intended to make disulfide bonds in the oxidative endoplasmic reticulum environment, and the known observation that frog Tg is highly glycosylated and sulfated (a Golgi/post-Golgi activity) and secreted, and the fact that at a time when Tg is expressed, colloid protein begins to fill the lumen of thyroid follicles. We then used the SignalP signal peptide prediction software which indicated no potential signal peptide contained within our *Xenopus* Tg cDNA sequence. This result was also surprising because our analysis of the lamprey Tg coding sequence revealed the presence of an excellent predicted signal peptide (MRTSPLLPATTTLYLVLWIGTISA-LYSDS).

To test the potential secretability of the *Xenopus* Tg protein, we replaced the 5' sequence of our *Xenopus* Tg cDNA with with that encoding a functional signal peptide. In multiple replicate clones, introduction of the mouse Tg signal peptide resulted in improved expression and secretion of the *Xenopus* Tg protein (Figure 7). Thus, there is a high likelihood that there is either a sequencing error or an mRNA splicing event that can account for the absence of the *Xenopus* Tg signal peptide in our predicted sequence, and thus a high likelihood that *Xenopus* Tg protein is a functional TH precursor protein.

Discussion

In this paper we characterized Tg sequences in *Xenopus* and zebrafish and we examined the conservation of the *Tg* gene at the scale of vertebrates. We did not found any protein orthologous of the Tg among non-vertebrate species, strongly suggesting that Tg is a vertebrate-specific protein. We observed that the different domain of the Tg are conserved in vertebrates. Although the overall amino acid sequences are quite divergent between human, *Xenopus*, zebrafish and lamprey, some important residues (e.g. the disulfide bond forming cysteins and the hormonogenic tyrosines) are extremely well conserved. These

conservations of both domain and cystein are consistent with the secondary structure predictions that are similar between human, *Xenopus*, zebrafish and lamprey. The secretion properties of *Xenopus* and zebrafish Tg are similar to those of mammal proteins. Together, these observations makes Tg evolution unusual since it exhibit few very specific amino acids highly conserved in an ocean of sequence divergence which is enough to ensure a conservation of structure and function.

Key functional residues conserved in vertebrate Tg

Despite the fact that overall Tg sequences are quite divergent (only 14% amino acid conservation between the four Tg proteins studied here) some specific amino acids of the Tg sequences are remarkably conserved.

The first clear examples are the hormonogenic sites. The tyrosines at the orthologous position of human Y5 and Y130 are strikingly conserved in the Tg of all species known to date. It is known that those two residues help to form the major hormogenic site in mammals, with a preference for T4 (Palumbo *et al.*, 1990; Dunn & Dunn, 1999). Those two tyrosines, but not the tyrosine in between, are conserved, enforcing the idea that they are likely to work together in all vertebrates. Given the 3D structure imposed by the cysteines and the resulting disulfide bonds, it is most certainly possible that the Tg folds in a way that other tyrosines between Y5 and Y130 cannot be used to synthesize TH with the same efficiency. Thus, mutation of either Y5 or Y130 would likely be counter-selected as this would adversely affect hormone production. Of course, it is conceivable that mutation could occur simultaneously in a cysteine as well as a new neighbouring hormonogenic tyrosine, producing secondary sites of TH formation that are not present in ancestral Tg molecules.

In addition to Y5 and Y130 the last two tyrosines, Y2554 and Y2747 orthologs, are also conserved in the species we studied. Even if the Y2554 falls into the unsequenced part of the lamprey Tg gene, the orthologs are conserved in *Xenopus* and zebrafish, suggesting that these two tyrosines are *bona fide* hormonogenic sites. This contrasts with the internal hormonogenic tyrosines, namely Y857, Y1291 and Y1448, which are poorly conserved in the species we studied. This absence of conservation is consistent with biochemical evidence suggesting that in mammals, those tyrosines are not the most widely used for TH synthesis (Ohmiya *et al.*, 1990). Tg is known to undergo conformational changes, especially under low

TSH and high TSH conditions(Berg & Ekholm, 1975; Dunn & Ray, 1975) . It is therefore possible that these conformational changes are used to maximize hormone production in the absence of iodine and it may be possible that in such situation different hormonogenic sites are used.

A second important class of conserved residues are the cysteine, of which 90% are found at the same positions between human, *Xenopus*, zebrafish and lamprey. These amino acids ,are known to form intra-domain disulfide bonds important for Tg conformation. Given the complex trafficking of the Tg between the thyrocyte and the lumen of the thyroid gland, it is clear that the 3D structure of the protein must be tightly controlled and therefore conserved. Interestingly, most cysteine loss observed between Human, *Xenopus*, zebrafish and lamprey corresponds in fact to loss of pairs of cysteines that form a specific disulfide bounds. This is the case of cysteines at position 1012/1021 in *Xenopus* and 114/120, 1146/1166 and 1558/1569 in lamprey. Note that evolutionary absence of one cysteine would mean the loss of a specific disulfide bond, and this is expected to relieve selection pressure on the other cysteine, allowing its mutation. Why some specific disulfide bonds are less important in Tg will have to await a 3D structural analysis of the Tg protein.

Conservation of the domains in vertebrate Tg

It is quite striking that the domain composition of Tg is very much conserved suggesting a strong selective pressure on the overall organization of the protein. For example both the hinge and the linker that separate the Tg repeats are present in all the studied proteins. These domains do not harbour major insertion or deletion, suggesting that they have an important role in protein structure. The linker has only two cysteins but it is predicted to be formed at 30-35% of helixes and 6-10% of beta sheets, with a conservation of these structure between the 4 sequences. On the other hand, the hinge harbour 11 conserved cysteins and is formed at 35% of coiled sequence, 10% of helixes and 6% of beta sheet for the four sequences (Figure S1). Together, this indicates that this region is structured, rather than representing an uncoiled domain between Tg1-10 and Tg2-1 repeats. This also applies within the Tg repeats: not only are the 3 types of Tg repeat present in the Tg all vertebrates, but also their number and order from N-terminal to C-terminal is consistent. Interestingly within repeat Tg1-3, Tg1-7 and Tg1-8 (Malthiéry and Lissitzky 1987, figure S1) there are

also additional sequences of consistent size, lacking cysteines, that are present in human, *Xenopus*, zebrafish and lamprey, suggesting that these regions are also necessary for the correct folding of the Tg protein or the interaction with other protein during TH synthesis The strong conservation of Tg in lamprey when compared to the gnathostome Tgs is noticeable given the divergence in thyroid gland organization of the lamprey, and the fact that the two clades diverged 500 million years ago (Smith *et al.*, 2013). Indeed, in the pharyngeal area of the lamprey larva (the ammocoete) there is an exocrine secretion of TH (Youson, 1997) via the endostyle that may considered as the equivalent of the thyroid gland since it expresses the *TTF1/Nkx-2.1*, a transcription factor that, along with *Pax-8*, is required for thyroid specification(Kimura, 1996) . During metamorphosis, the endostyle becomes reorganized into a true endocrine thyroid gland (Suzuki & Kondo, 1973; Youson, 1997; Kluge *et al.*, 2004). As the immunolabeling of lamprey Tg remains controversial because of possible cross-reactivity of the antibody (Kluge *et al.* 2005), and since no Tg has been cloned in lamprey, it remains unclear whether Tg expression starts in the ammocoete or in the adult when the gland is fully formed. This point is critical to understand the evolution of Tg in vertebrates specifically because a very similarly organized endostyle, harbouring *TTF1/Nkx-2.1* expression (Ogasawara *et al.*, 2001), is also found in invertebrate chordates such as amphioxus(Hiruta *et al.*, 2005) . Strikingly there is no trace of the Tg gene in amphioxus, raising the question of whether the Tg gene we identified in lamprey is effectively expressed in amnocoete and responsible for TH synthesis in the endostyle.

It is worth noting that, while Tg1 repeat, ChEL domain, and to a lesser extent Tg1 can be found in other proteins such as nidogen, the hinge and linker domains do not have any equivalent in other known proteins. This calls for a deeper investigation of the biological role of those two domains especially given that they articulate between well distinct domains of the Tg, namely the Tg1 repeats, the Tg2/Tg3 repeat and the ChEL domain. These domains could also be crucial to understand the evolution of the Tg given that they do not look like any functional domain of other proteins.

How has Tg evolved?

If Tg is conserved among vertebrates, it is striking that we were not able to identified any orthologs of Tg outside of vertebrates where TH is known to be important, such as in amphioxus (Paris *et al.*, 2008) or in *Ciona* (Patricolo *et al.*, 2001). Thus, it is very puzzling to wonder how such a protein could be found in the vertebrate ancestor but not earlier. One puzzling question is therefore the synthesis of TH outside vertebrates. Indeed, if it is known that TH pathway is functional outside vertebrates, we do not know how these organisms synthesize their TH. Two hypotheses can be made. (i) A protein with tyrosine, ancestral of Tg or not, is used to synthetize TH by the conjugation of two iodinated tyrosines as we observe in vertebrate. (ii) TH is made from iodinated compounds taken in food. This hypothesis might be supported by the presence of iodine compounds in the endostyle of ammocoete which primary function is to trap food (Fujita & Honma, 1969).

All appears as if the Tg design is a vertebrate-specific novelty that was set up at the basis of this phylum and has been only very marginally modified since then. A peculiarity of vertebrates that is likely to explain the presence of Tg is the thyroid gland itself. Indeed, Tg undergoes a complex trafficking from the thyrocyte to the follicular lumen and the correct folding of Tg is essential for that trafficking. Thus, without a gland-like organization, such a highly conserved Tg structure would not likely have been selected. It would therefore be interesting to assess the question of the co-evolution of Tg and the gland. Given what happens during its development, lamprey seems to be the keystone of the understanding of Tg evolution, since as mentioned before, the larvae endostyle become the adult thyroid gland after metamorphosis (Youson, 1997). Pushing this hypothesis further, the need for a higher or a more controlled quantity of TH during vertebrate life time could explain the presence of the Tg. Indeed, the protein, as a scaffold dedicated to TH synthesis, might have been selected to ensure a continued fine-tuned supply of TH resistant to changes in iodine availability.

Alternatively, given the modular structure of Tg, and because the cysteines form disulfide bonds only within the same Tg repeats, it is possible to suggest a speculative step-by-step process that lead to the observed Tg. One hypothesis could be that an ancestral protein harbouring Tg repeat-like structure, able to synthetize TH but not necessarily selected for that, was mis-spliced and accumulate the Tg repeat in the vertebrate ancestral lineage. This hypothesis could explain the origin of the linker and hinge domains allowing intronic sequence to become expressed as part of the Tg coding sequence. In the vertebrate lineage,

this ancestral protein would have reached a certain threshold of complexity and involvement in TH synthesis that would prevent it for being lost. Thus starting from a protein with low efficiency, selective forces would have favoured the emergence of the Tg as we know it. What is striking however is that we have so far no evidence for the existence of such early divergent Tg precursor since, in all genomes analyzed to date Tg seems to appear well formed at once, with no obvious step-by-step process

These two non-exclusive hypothesis (co-evolution of Tg/thyroid and the step by step process) have in common that at some point some selective pressure would have push for the selection for a fine-tuned system of TH synthesis in the vertebrate lineage but not in others lineages. The reason for such a push remains obscure and can't be discussed further without experimental evidence.

Conclusion

Tg is a peculiar protein with a highly conserved structure and no orthologs outside of vertebrates. Given its conservation among vertebrates (and the energetic investment involved in synthesizing >2700 amino acids per monomer), we can conclude that there are strong selective forces for its preservation. The cloning of the *Xenopus* and zebrafish Tg and the analysis of a lamprey Tg sequence have revealed an unexpected conservation with all domains being conserved across vertebrates. This also sheds light on the conserved biochemical properties of vertebrate Tg. Understanding the evolution of this protein in conjunction with TH synthesis will require investigating the lamprey Tg expression pattern and its biochemistry, particularly at metamorphosis during the ammocoete/juvenile transition. In addition, it will bring some clues to understand how TH is synthesized in invertebrate chordates.

Figures Legend

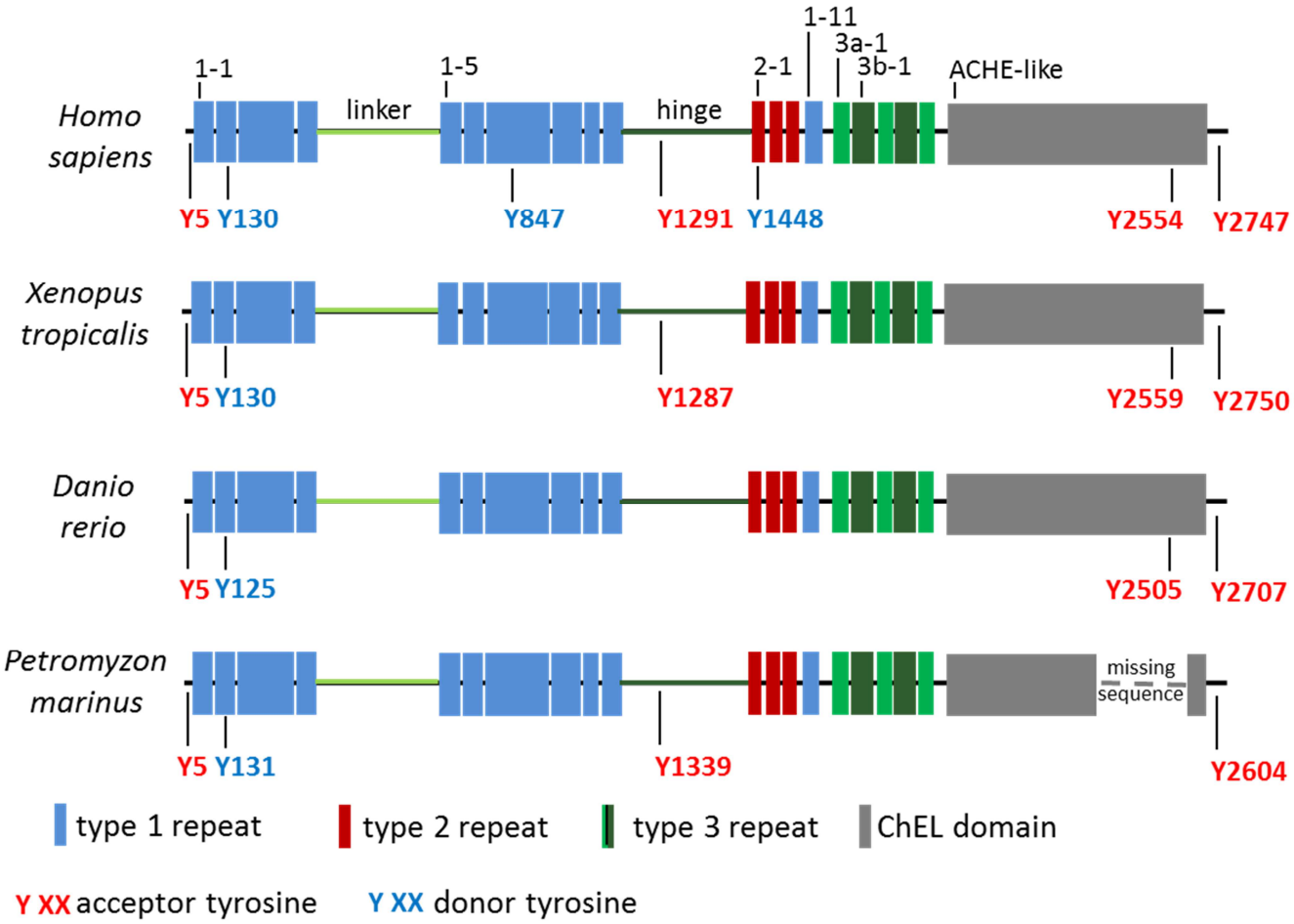


Figure 1: Schematic organization of human, *Xenopus*, zebrafish and lamprey Tgs. Tg1 repeats are shown by blue boxes, Tg2 repeats by red boxes, Tg3 repeat by green boxes and ChEL domain by grey boxes. The linker and the hinge are represented by a light green and dark green line respectively. The main hormogenic tyrosines are represented with their positions in each sequence, acceptor tyrosine are in red and don't tyrosine in blue.

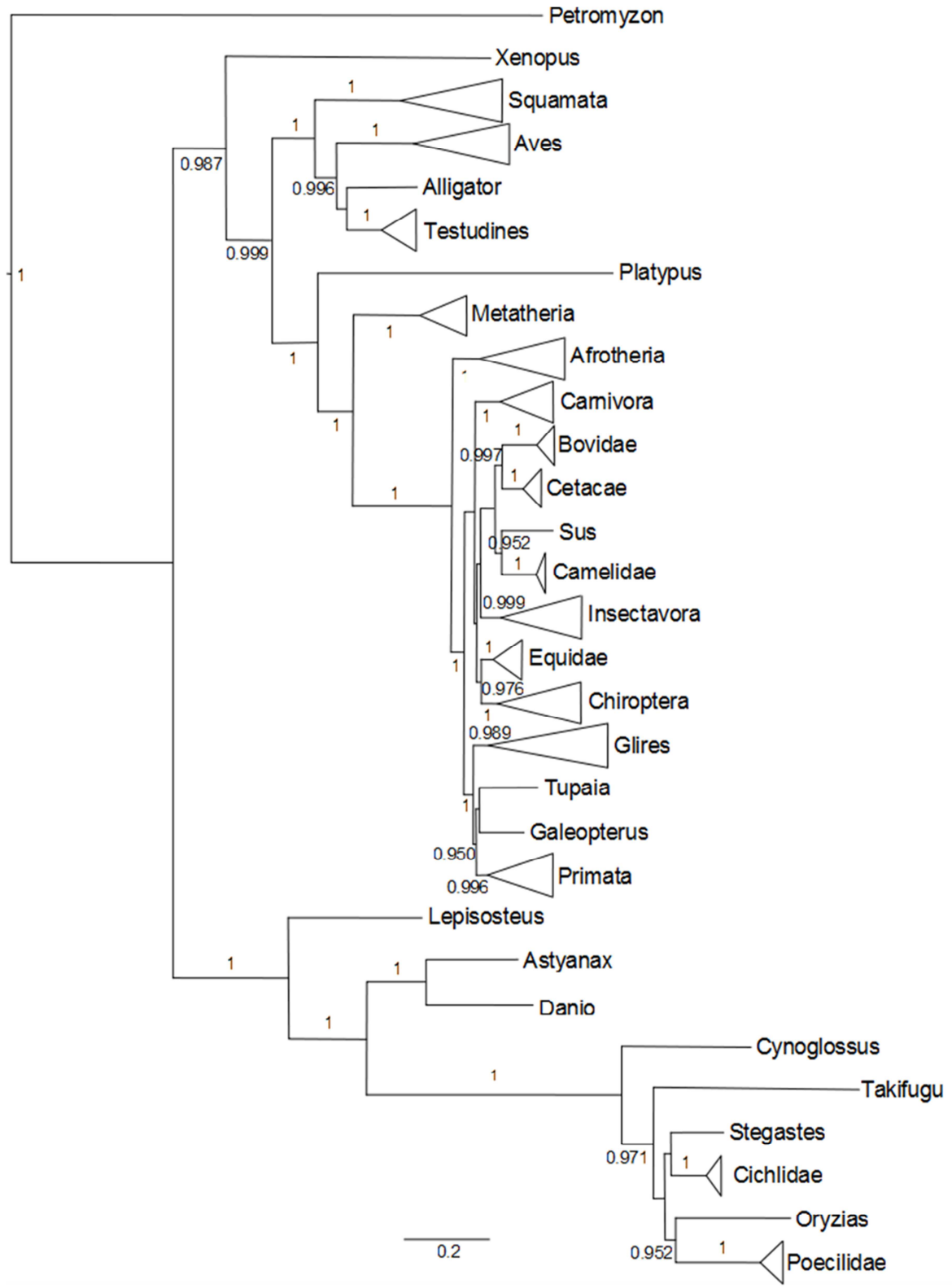


Figure 2: Phylogenetic reconstruction of thyroglobulins. The tree are built by Maximum likelihood containing both experimentally determined sequences as well as predicted sequences. A statistical SH-like branch support were computed and are indicated when >0.7.

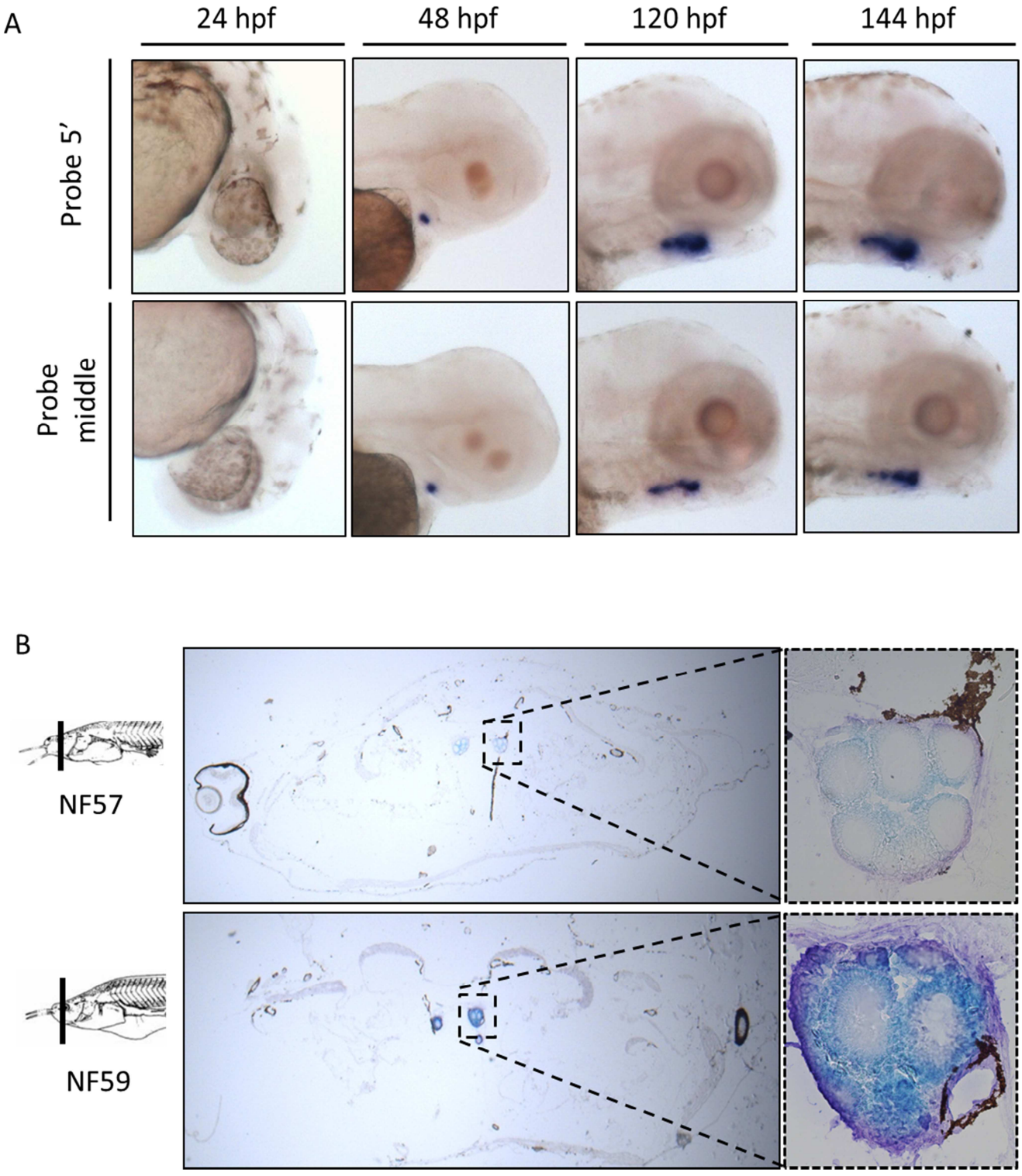


Figure 3: Expression of Tg in zebrafish (A) and *Xenopus* (B) revealed by *in situ* hybridization. (A) Whole zebrafish embryo, two probes were used for zebrafish: in the 5' part of the *tg* (upper panel) and in the middle (lower panel).ISH were performed at 24, 48, 120 and 144 hpf as indicated. (B) *Xenopus* tadpole sections, probe were used for xenopus at the stages NF57 and NF59.

A

Species	Database	Accession number	Status	Publication
Homo sapiens	NCBI	NM_003235.4	Cloned	Lamas et. al. 1989
Mus musculus	NCBI	AAB53204.1	Cloned	Direct submission 1996
Rattus norvegicus	NCBI	AAF34909.1	Cloned	Kim et. al. 2000
Canis familiaris	NCBI	NP_00104a1569.1	Cloned	Lee et. al. 2007
Sus scrofa	NCBI	ACY66900.1	Cloned	Direct submission 2009
Bos taurus	NCBI	NM_173883.2	Cloned	Hou et al. 2012

B

Species	Database	Accession number	Specie	Database	Accession number
Aeniopygia guttata	NCBI	XP002188056.2	*Melursus sinus*	NCBI	CAO02459.1
Ailuropoda melanoleuca	NCBI	XP002917658.1	*Mesitornis unicolor*	NCBI	KFQ37012.1
Alligator sinensis	NCBI	XP006022057.1	*Microtus ochrogaster*	NCBI	XP005354594.1
Anas platyrhynchos	NCBI	XP005010631.1	*Monodelphis domestica*	NCBI	XP003340716.2
Anolis carolinensis	NCBI	XP008106404.1	*Mustela putorius*	NCBI	XP004743644.1
Aptenodytes forsteri	NCBI	XP009272259.1	*Myotis brandtii*	NCBI	XP005872859.1
Astyanax mexicanum	NCBI	XP007249893.1	*Myotis davidii*	NCBI	XP006753353.1
Balaenoptera musculus	NCBI	XP007188966.1	*Myotis lucifugus*	NCBI	XP006093377.1
Bos mutus	NCBI	XP005900803.1	*Nannospalax galili*	NCBI	XP008841410.1
Bubalus bubalis	NCBI	XP006079406.1	*Neolamprologus multifasciatus*	NCBI	XP006805674.1
Buceros rhinoceros	NCBI	KFO85123.1	*Nipponia nippon*	NCBI	XP009468077.1
Callithrix jacchus	NCBI	XP008981504.1	*Nomascus leucogenys*	NCBI	XP003255990.2
Callorhinchus milii	NCBI	XP007888581.1	*Ochotona princeps*	NCBI	XP004580794.1
Calypte anna	NCBI	XP008503581.1	*Octodon degus*	NCBI	XP004642544.1
Camelus ferus	NCBI	XP006189268.1	*Odobenus rosmarus*	NCBI	XP004414052.1
Capra hircus	NCBI	XP005688888.1	*Opisthocomus hoazin*	NCBI	KFR16272.1
Cathartes aura	NCBI	KFP54291.1	*Orcinus orca*	NCBI	XP004265356.1
Cavia porcellus	NCBI	XP003467392.1	*Oreochromis niloticus*	NCBI	XP005456159.1
Ceratotherium simum	NCBI	XP004431111.1	*Ornithorhynchus anatinus*	NCBI	XP007661867.1
Chaetura pelagica	NCBI	KFU93110.1	*Orycteropus afer*	NCBI	XP007938731.1
Charadrius vociferus	NCBI	KGL90055.1	*Oryctolagus cuniculus*	NCBI	XP008254187.1
Chelonia mydas	NCBI	XP007066827.1	*Oryzias latipes*	NCBI	XP004078592.1
Chinchilla lanigera	NCBI	XP005398080.1	*Otolemu rgarnettii*	NCBI	XP003792914.1
Chlorocebus sabaeus	NCBI	XP007999775.1	*Ovis aries*	NCBI	XP004011680.1
Chrysemys picta bellii	NCBI	XP008163419.1	*Pan paniscus*	NCBI	XP003830134.1
Chrysochloris asiatica	NCBI	XP006830780.1	*Pan troglodytes*	NCBI	XP003311969.1
Columba livi	NCBI	XP005510877.1	*Panthera tigris*	NCBI	XP007073886.1
Condylura crista	NCBI	XP004680035.1	*Pantholops hodgsonii*	NCBI	XP005973929.1
Corvus brachyrhynchos	NCBI	XP008635720.1	*Papio anubis*	NCBI	XP003903225.1
Cricetulus griseus	NCBI	XP007632427.1	*Pelecanus crispus*	NCBI	XP009478908.1
Cuculus canorus	NCBI	XP009558342.1	*Pelodiscus sinensis*	NCBI	XP006123599.1
Cynoglossus semilaevis	NCBI	XP008321228.1	*Peromyscus maniculatus*	NCBI	XP006980379.1
Echinops Telfair	NCBI	XP004697442.1	*Petromyzon marinus*	Ensembl	ENSPMAG00000001187
Egretta garzetta	NCBI	XP009646261.1	*Phalacrocorax carbo*	NCBI	XP009510665.1
Elephantulus edwardii	NCBI	XP006879337.1	*Phoenicopterus ruber*	NCBI	KFQ84232.1
Eptesicus fuscus	NCBI	XP008154848.1	*Physeter catodon*	NCBI	XP007106504.1
Equus caballus	NCBI	XP001916622.1	*Picoides pubescens*	NCBI	KFV61751.1
Equus przewalskii	NCBI	XP008513751.1	*Poecilia formosa*	NCBI	XP007577005.1
Erinaceus europaeus	NCBI	XP007521963.1	*Poecilia reticulata*	NCBI	XP008428883.1
Eurypyga helias	NCBI	KFV90909.1	*Pongo abelii*	NCBI	XP009242382.1
Falco cherrug	NCBI	XP005441229.1	*Pseudopodoces humilis*	NCBI	XP005517435.1
Falco peregrinus	NCBI	XP005237290.1	*Pteropus alecto*	NCBI	XP006912991.1
Felis catus	NCBI	XP004000173.1	*Pundamilia nyere*	NCBI	XP005744254.1
Ficedula albicollis	NCBI	XP005042607.1	*Pygoscelis adeliae*	NCBI	XP009321658.1
Fukomys damarensis	NCBI	KFO30974.1	*Python bivittatus*	NCBI	XP007426175.1
Fulmarus glacialis	NCBI	XP009580711.1	*Saimiri boliviensis*	NCBI	XP003933743.1
Galeopterus variegatus	NCBI	XP008568520.1	*Sarcophilu sharr*	NCBI	XP003760448.1
Gallus gallus	NCBI	XP003640916.2	*Serinus canaria*	NCBI	XP009089053.1

Geospiza fortis	NCBI	XP005425780.1
Gorilla gorilla	NCBI	XP004047607.1
Haplochromis burtoni	NCBI	XP005941141.1
Helarctos malayanus	NCBI	CAO02458.1
Heterocephalus glaber	NCBI	EHB17216.1
Ictidomys tridecemlineatus	NCBI	XP005316198.1
Jaculus jaculus	NCBI	XP004656389.1
Latimeria chalumnae	NCBI	XP006009656.1
Lepisosteus oculatus	NCBI	XP006635897.1
Leptonychotes weddellii	NCBI	XP006749666.1
Leptoso musdiscolor	NCBI	KFQ06856.1
Lipotes vexillifer	NCBI	XP007448059.1
Loxodonta africa	NCBI	XP003408346.1
Macaca fascicularis	NCBI	EHH64438.1
Macaca mulatta	NCBI	EHH28780.1
Manacus vitellinus	NCBI	XP008933017.1
Maylandia zebra	NCBI	XP004571173.1
Meleagris gallopavo	NCBI	XP003205346.1
Melopsittacus undulatus	NCBI	XP005143880.1
Sorex araneus	NCBI	XP004602656.1
Stegastes partititus	NCBI	XP008304814.1
Struthio camelus	NCBI	XP009667772.1
Takifugu rubripes	NCBI	XP003965808.1
Tarsius syrichta	NCBI	XP008060148.1
Tetraodon nigroviridis	NCBI	CAF94496.1
Tinamus guttatus	NCBI	KGL75755.1
Tremarctos ornatus	NCBI	CAO02461.1
Trichechus manatus	NCBI	XP004373071.1
Tupaia chinensis	NCBI	XP006155589.1
Tyto alba	NCBI	KFV40071.1
Ursus americanus	NCBI	CAO02457.1
Ursus arctos	NCBI	CAO02456.1
Ursus maritimus	NCBI	XP008682382.1
Ursus thibetanus	NCBI	CAO02460.1
Vicugna pacos	NCBI	XP006208079.1
Xiphophorus maculatus	NCBI	XP005813762.1
Zonotrichia albi	NCBI	XP005479784.1

Supplementary Table 1:(A) list of cloned Tg genes available on genebank with the related paper. (B) list of predicted Tg genes from various databases. The predicted sequences were retrieved from a blast of human Tg against the database and manually curated.

Species	Primer	Step	Sens	Sequence (5'-3')
Xenopus tropicalis	internal nested	5' end cloning	reverse	CTGCTTCCTGCTAATTCTGCC
Xenopus tropicalis	external nested	5' end cloning	reverse	ATCTCCACTGCACTGTATGTTTCTG
Xenopus tropicalis	F1	i	forward	CCTCTGCGTCCTTGTGAGCTG
Xenopus tropicalis	F2	i	forward	CGCGGAGTTGCCAAGTAAGAGG
Xenopus tropicalis	F3	i	forward	ATGTCATTTCATGAGGATTCGACC
Xenopus tropicalis	F4	i	forward	GCAGAAGCTGTACAAGTCGTCG
Xenopus tropicalis	F5	i	forward	GCACAGTTCCAGCTCCTTCTTCC
Xenopus tropicalis	F6	i	forward	CGACTGGAGTGGAACGTCCC
Xenopus tropicalis	F7	i	forward	CTACGGCCTGCGGAACCATCAG
Xenopus tropicalis	R1	i	reverse	CCATGTCCCTGACAAAGCTGC
Xenopus tropicalis	R2	i	reverse	CGCTGTACATGCCCGTTAATCCG
Xenopus tropicalis	R3	i	reverse	CACTGCGGGACATAAACATCGG
Xenopus tropicalis	R4	i	reverse	CCATTAAACTGGCCGGTCTTGG
Xenopus tropicalis	R5	i	reverse	GCAATCATCCGGGGTCAGACTG
Xenopus tropicalis	R6	i	reverse	CCTCGAAGGCAGGTCAGAAGATC
Xenopus tropicalis	R7	i	reverse	TCACAGCTGGAACTCTCCTGTAGAC
Xenopus tropicalis	3'UTR	i	reverse	TCACAGCTGGAACTCTCCTC
Xenopus tropicalis	I	ii	forward	**ACTATAGGGCGAATTC** TGGTCTGTACCATAGTCATCATC
Xenopus tropicalis	II	ii	forward	GAGTTATTTATTCCAGCCTGTGATC
Xenopus tropicalis	III	ii	forward	CCTTCATGCCCAGTTTACACG
Xenopus tropicalis	IV	ii	forward	CAATCAGCGGTCTCCTCCATC
Xenopus tropicalis	I	ii	reverse	ATCACAGGCTGGAATAAATAACTC
Xenopus tropicalis	II	ii	reverse	CGTGTAAACTGGGCATGAAGG
Xenopus tropicalis	III	ii	reverse	GATGGAGGAGACCGCTGATTG
Xenopus tropicalis	IV	ii	reverse	**CTGCTTTAATAAGATCT** TCACAGCTGGAACTCTCCTC
Danio rerio	ATG	i	forward	gATGCAGAACATGGAAGAGATGA
Danio rerio	F1	i	forward	GACTCATCAGCAGAGCCAAGAAC
Danio rerio	F2	i	forward	GAGCTGACATTGCGAGCCTTC
Danio rerio	F3	i	forward	GCAAAGACATAGCAGAGGACCTGG
Danio rerio	F4	i	forward	GCTGTTGGCGAGGATTCTAGAAGTG
Danio rerio	F5	i	forward	GCACAGGGGGAGGAACTTACTTG
Danio rerio	F6	i	forward	GCGCTGTGAGCTGTACTGTGAC
Danio rerio	F7	i	forward	GTATCTGAGCCCTGAGGAGCTG
Danio rerio	F8	i	forward	CTCTGATGCGTTTGGTCGATCGG
Danio rerio	F9	i	forward	GTACCGACAATGGGGCGTATGTG
Danio rerio	F10	i	forward	ATGTTCAGTGTAACCGTGGAGG
Danio rerio	R1	i	reverse	CCTTGTAGCTGAAGAGGTCACGC
Danio rerio	R2	i	reverse	GAGGAACTCCGAAGAGATACTGCAC
Danio rerio	R3/Stop	i	reverse	GCAGAGACTGCATTGGTTTTAAGC
Danio rerio	R4	i	reverse	GGCTCTGCTGATGAGTCCATCC
Danio rerio	R5	i	reverse	CCAAGAAGTAAGTCACACGCTTGC
Danio rerio	R6	i	reverse	CACATGCAAAATACAGAGGGCCTTC
Danio rerio	R7	i	reverse	CTCAAATCTCTTCAGGCCCGCTG
Danio rerio	R8	i	reverse	CCTGTAGCTCTCATGTCTCTCAGC
Danio rerio	R9	i	reverse	CTGAGGTAGTGAGGAACGAGACGTC
Danio rerio	R10	i	reverse	GGATCCAGTGGTGCGAGAACC
Danio rerio	I	ii	forward	**CACTATAGGGCGAATTC** CCTAGAGAAGATGCAGAACATG
Danio rerio	II	ii	forward	TGGCTCTTGAGAATACGCA
Danio rerio	III	ii	forward	CTCCAGTCCTCCTTCATTTAGC
Danio rerio	IV	ii	forward	ATTCACCTCTGTGTCCATCC
Danio rerio	I	ii	reverse	TGCGTATTCTCAAGAGCCA
Danio rerio	II	ii	reverse	GCTAAATGAAGGAGGACTGGAG
Danio rerio	III	ii	reverse	GGATGGACACAGAGGTGAAT
Danio rerio	IV	ii	reverse	**CTGCTTTAATAAGATCT** GTTTTAAGCAATTACAAAAAAAC

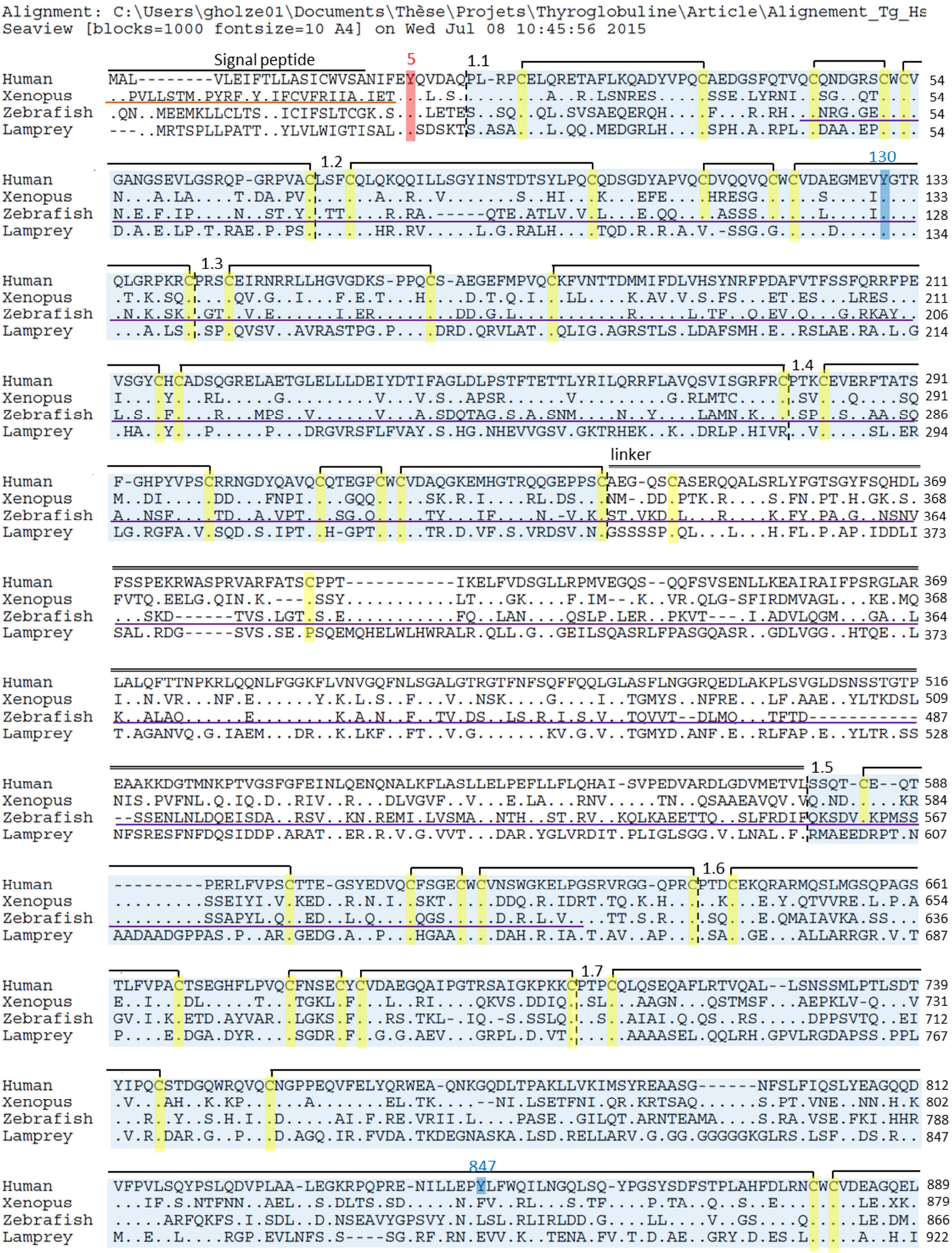
Supplementary Table 2: List of primer used to clone the *Xenopus* (upper) and zebrafish (lower) Tg. The third column gives the step of the cloning: (i) sequencing, (ii) cloning. The primer I forward and IV reverse for each species were used to clone the gene into the plasmid by recombination. The sequences homologous to the cloning plasmid appear in bold.

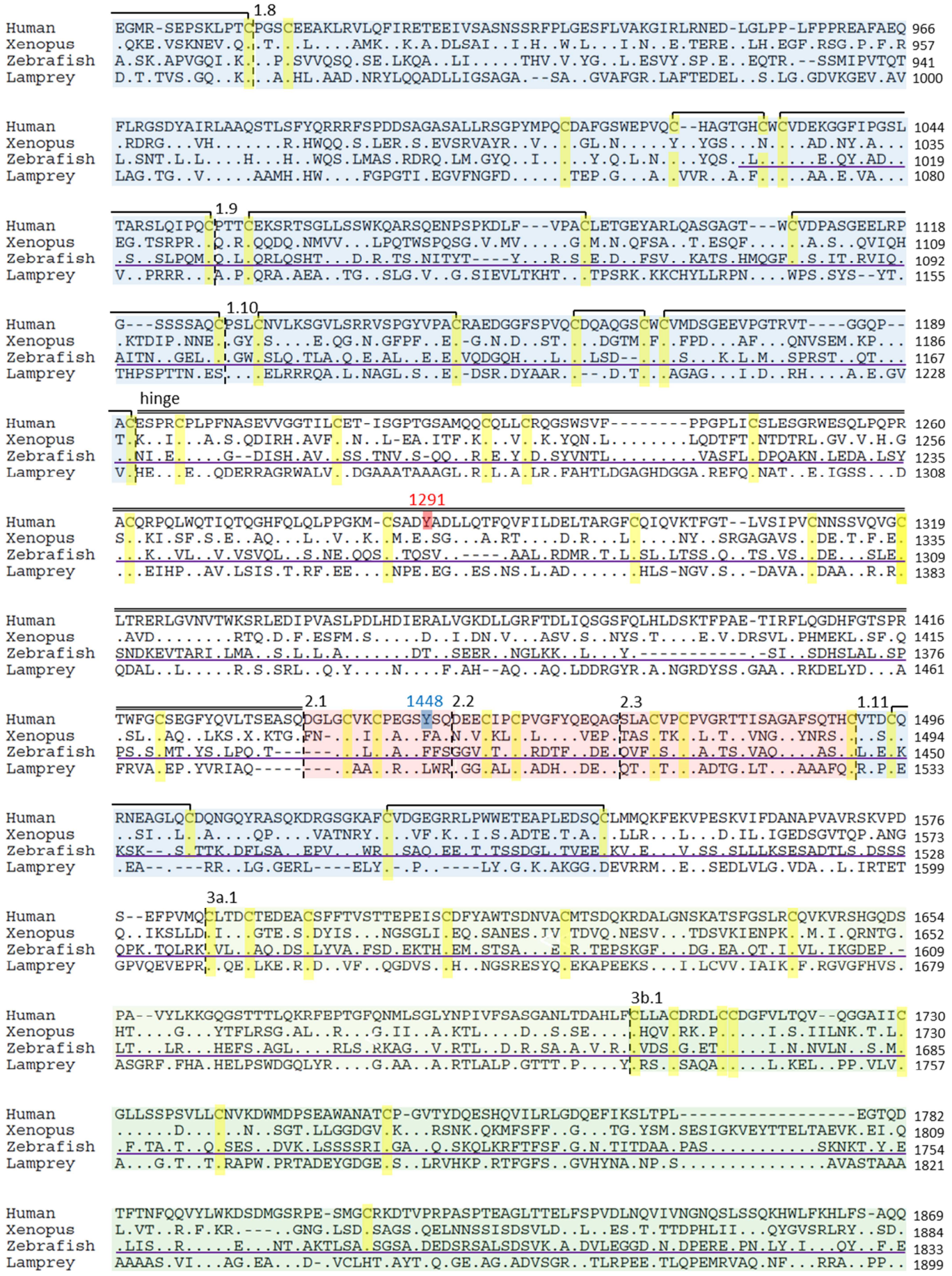
Taxa	Species
Tunicate	*Ciona intestinalis*
Tunicate	*Oikopleura dioica*
Tunicate	*Oikopleura vanhoeffeni*
Tunicate	*Oikopleura albicans*
Tunicate	*Oikopleura longicauda*
Tunicate	*Mesochordaeus bahamasi*
Tunicate	*Bathochordaeus charon*
Tunicate	*Fritillaria borealis*
Cephalochordate	*Branchiostoma floridae*
Echinoderm	*Strongylocentrotus purpuratus*
Hemichordate	*Saccoglossus kowalevskii*
Mollusk	*Crassostrea gigas*
Annelid	*Capitella teleta*
Cnidaria	*Nematostella vectensis*
Xenocoelomorph	*Xenoturbella bocki*
Xenocoelomorph	*Symsagittifera roscoffensis*
Xenocoelomorph	*Paraphanostoma dubium*
Xenocoelomorph	*Nemertoderma westbladi*
Xenocoelomorph	*Meara stichopi*
Xenocoelomorph	*Paratomella unichaeta*
Xenocoelomorph	*Isodiametra pulchra*

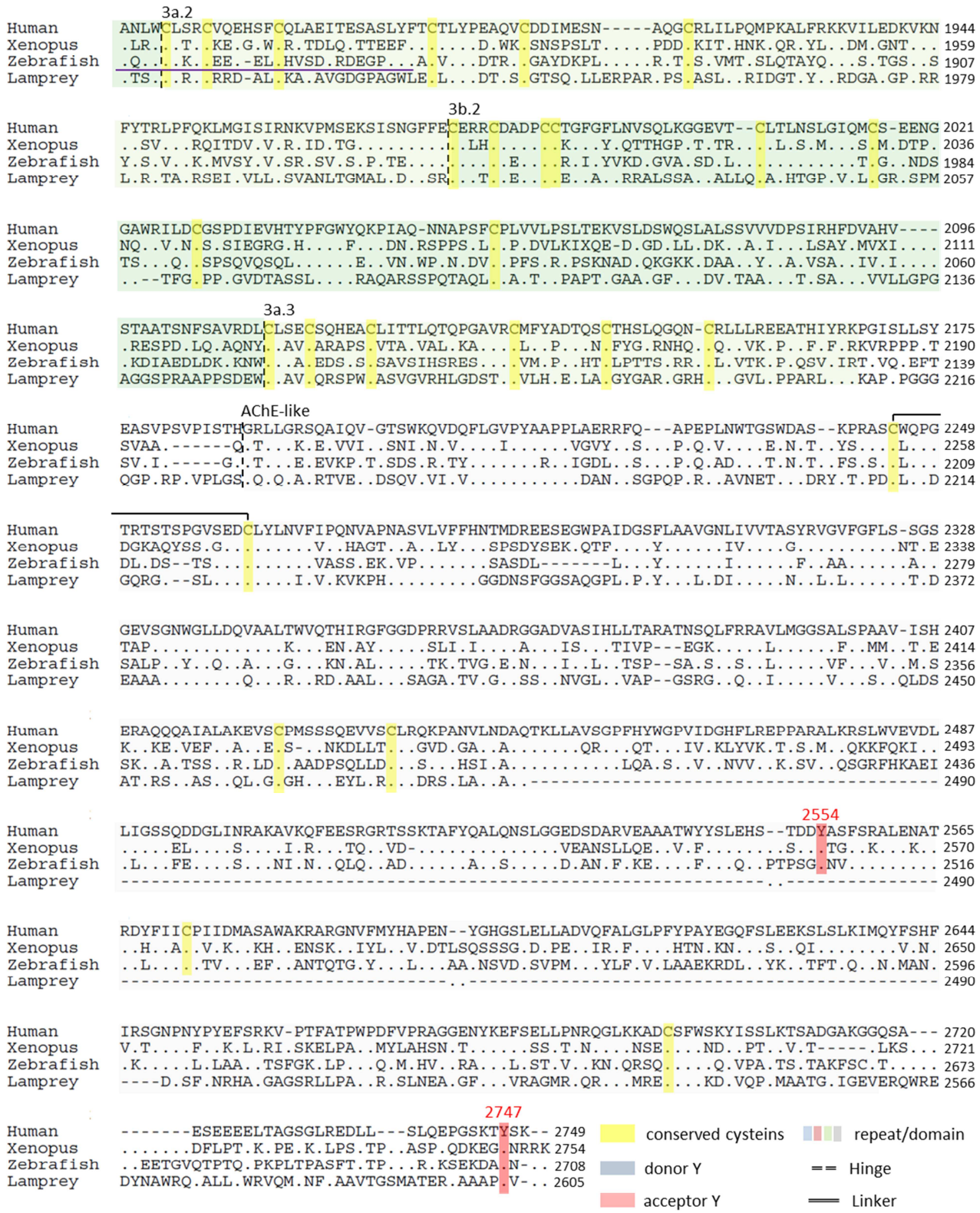
Supplementary Table 3: List of non-vertebrate species investigated for Tg. The Xenocoelomorph genomes were kindly investigated by Max Telford.

Protein	Species	Database	Accession number
SMOC1	*Homo sapiens*	NCBI	NP_001139689.1
SMOC1	*Mus musculus*	NCBI	Q9H4F8.1
SMOC1	*Danio rerio*	NCBI	ENSDARG00000088255
Nidogen	*Homo sapiens*	NCBI	CAA57709.1
Nidogen	*Mus musculus*	NCBI	AAI31670.1
Nidogen	*Danio rerio*	NCBI	XP_009296690.1
TACSTD	*Homo sapiens*	NCBI	CAG47055.1
TACSTD	*Mus musculus*	NCBI	NP_032558.2
TACSTD	*Danio rerio*	NCBI	NP_001017593.1|

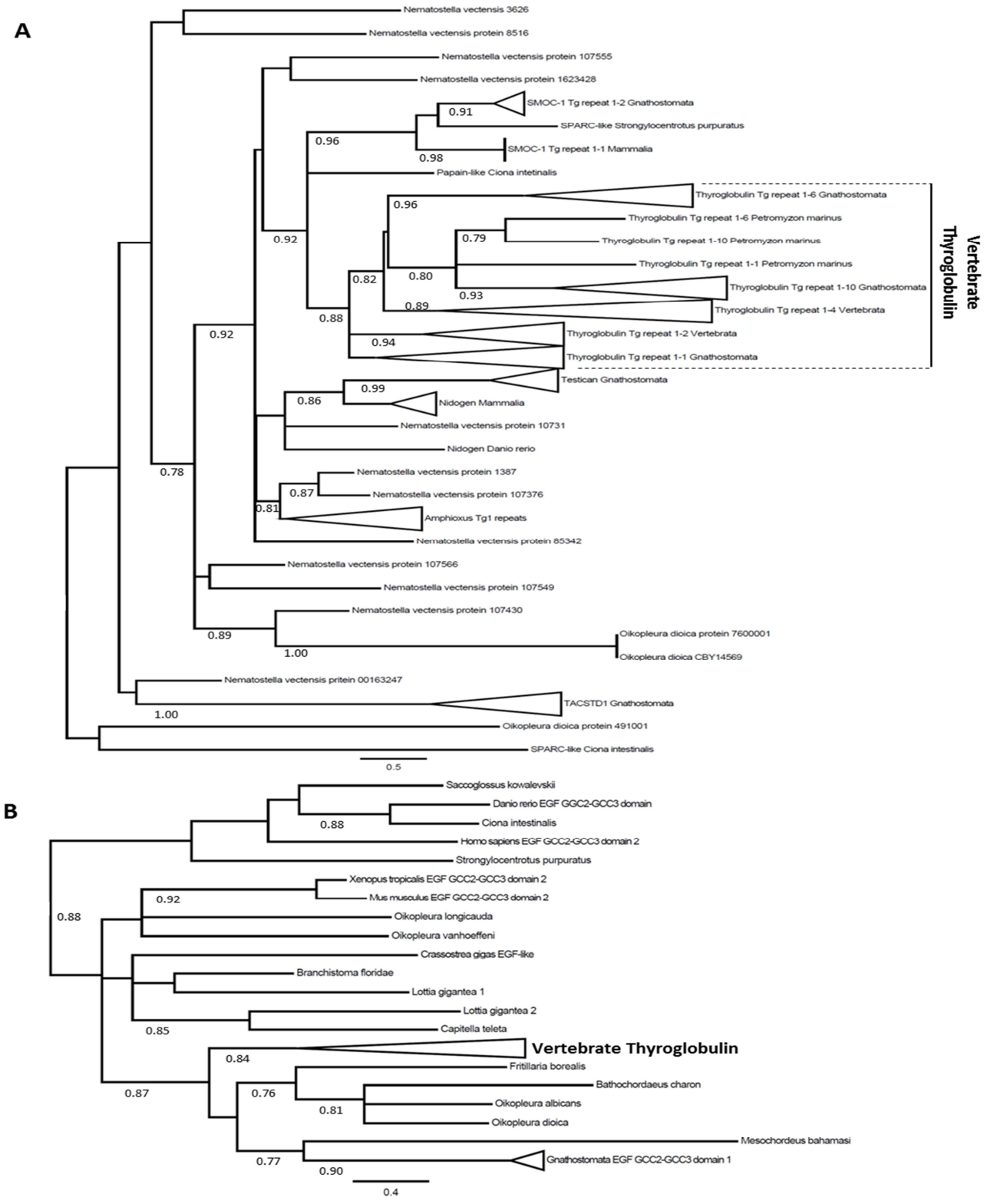
Supplementary Table 4: List of Tg repeat containing protein


Alignment: C:\Users\gholze01\Documents\Thèse\Projets\Thyroglobuline\Article\Alignement_Tg_Hs
Seaview [blocks=1000 fontsize=10 A4] on Wed Jul 08 10:45:56 2015
Signal peptide
5
1.1
1.2
130
1.3
1.4
linker
1.5
1.6
1.7
847
Human
Xenopus
Zebrafish
Lamprey

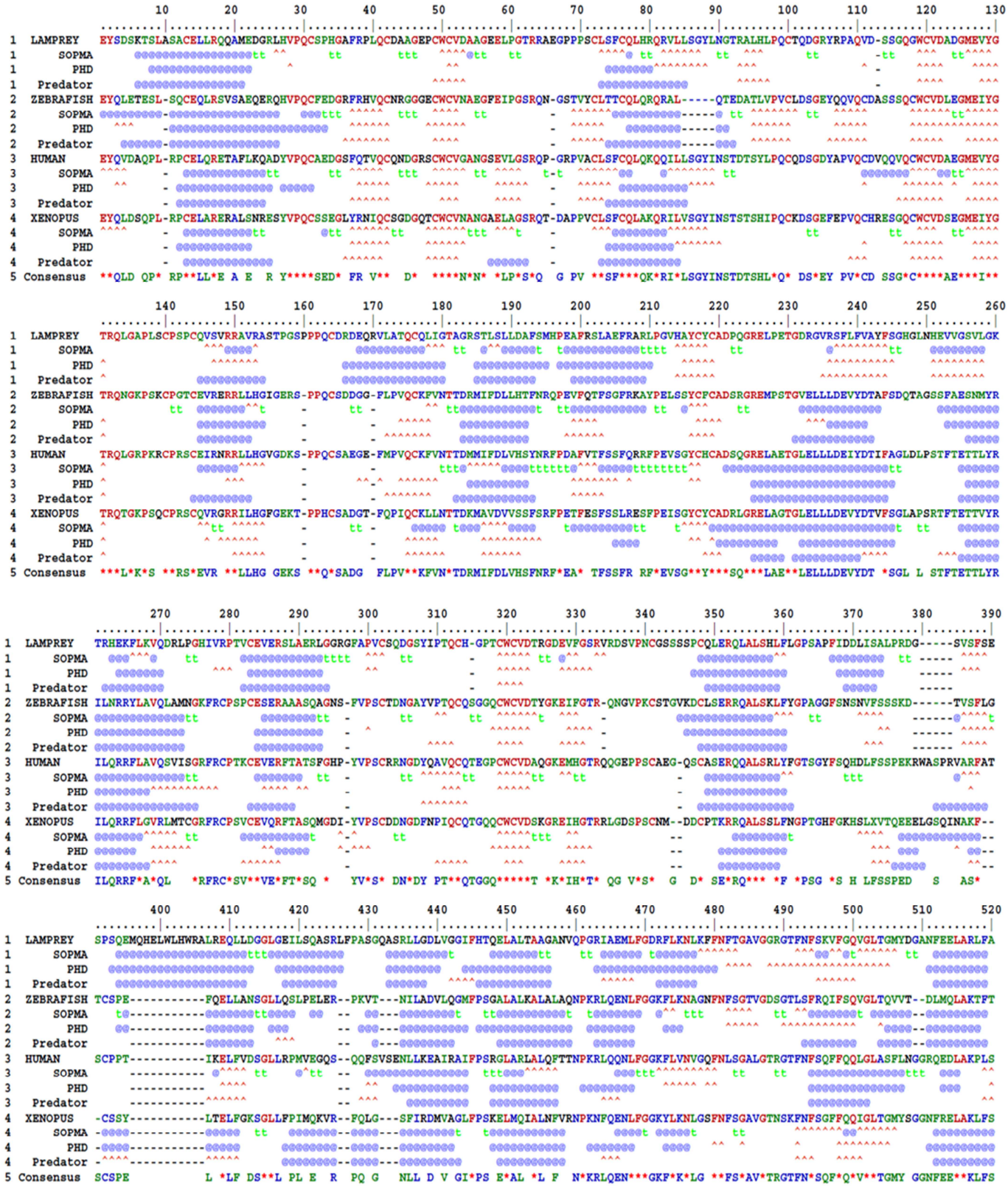

1.8
Human EGMR-SEPSKLPTCPGSCEEAKLRVLQFIRETEEIVSASNSSRFPLGESFLVAKGIRLRNED-LGLPP-LFPPREAFAEQ 966
Xenopus .QKE.VSKNEV.Q..T..L....AMK..K.A.DLSAI..I.H..W.L...I.N..E.TERE..LH.EGF.RSG.P.F.R 957
Zebrafish A.SK.APVGQI.K...P.SVVQSQ.SE.LKQA..LI.....THV.V.YG..L.ESVY.SP.E..EQTR.--SSMIPVTQT 941
Lamprey D.T.TVS.GQ..K...A.HL.AAD.NRYLQQADLLIGSAGA.--SA..GVAFGR.LAFTEDEL..S.LG.GDVKGEV.AV 1000
Human FLRGSDYAIRLAAQSTLSFYQRRRFSPDDSAGASALLRSGPYMPQCDAFGSWEPVQC--HAGTGHCWCVDEKGGFIPGSL 1044
Xenopus .RDRG...VH.....R.HWQQ.S.LER.S.EVSRVAYR..V....GL.N.....Y..YGS...N....AD.NY.A... 1035
Zebrafish L.SNT.L.L....H...H..WQS.LMAS.RDRQ.LM.GYQ..I.....Y.Q.L.N....YQS..L.....E.QY.AD.. 1019
Lamprey LAG.TG..V.....AAMH.HW....FGPGTI.EGVFNGFD.....TEP.G...A..VVR..A.F.....AA.E.VA... 1080
1.9
Human TARSLQIPQCPTTCEKSRTSGLLSSWKQARSQENPSPKDLF---VPACLETGEYARLQASGAGT---WCVDPASGEELRP 1118
Xenopus EG.TSRPR..Q.R.QQDQ.NMVV...LPQTWSPQSG.V.MV.....G.M.N.QFSA..T.ESQF.....A.S..QVIQH 1109
Zebrafish .S..SLPQM.Q.L.QRLQSHT...D.R.TS.NITYT----Y...R.S.E.D..FSV..KATS.HMQGF..S.IT.RVIQ. 1092
Lamprey V..PRRR...A.P.QRA.AEA..TG..SLG.V..G.SIEVLTKHT...TPSRK.KKCHYLLRPN....WPS.SYS--YT. 1155
1.10
Human G---SSSSAQCPSLCNVLKSGVLSRRVSPGYVPACRAEDGGFSPVQCDQAQGSCWCVMDSGEEVPGTRVT----GGQP-- 1189
Xenopus .KTDIP.NNE..GY.S....E.QG.N.GFPF..E.-G.N.D..ST....DGTM.F..FPD...AF...QNVSEM.KP... 1186
Zebrafish AITN..GEL..GW.SLQ.TLA.Q.E.AL..E.E.VQDGQH...L...LSD-------S...K.L.M..SPRST..QT... 1167
Lamprey THPSPTTN.ES....ELRRRQA.L.NAGL.S..E.-DSR.DYAAR..--.D.T...AGAG...I.D..RH....A.E.GV 1228
hinge
Human ACESPRCPLPFNASEVVGGTILCET-ISGPTGSAMQQCQLLCRQGSWSVF--------PPGPLICSLESGRWESQLPQPR 1260
Xenopus T.K..I...A.S.QDIRH.AVF..N..L-EA.ITF.K...V..K.YQN.L........LQDTFT.NTDTRL.GV.V.H.G 1256
Zebrafish ..NI.E.....G--DISH.AV..SS.TNV.S-QQ..R.E.Y.D.SYVNTL.........VASFL.DPQAKN.LEDA.LSY 1235
Lamprey V.HE...E..QDERRAGRWALV..DGAAATAAAGL.R.L.A.LR.FAHTLDGAGHDGGA.REFQ.NAT..E.IGSS...D 1308
1291
Human ACQRPQLWQTIQTQGHFQLQLPPGKM-CSADYADLLQTFQVFILDELTARGFCQIQVKTFGT--LVSIPVCNNSSVQVGC 1319
Xenopus S..KI.SF.S.E..AQ...L..V..K..M.E.SG...A.RT....D.R...L.....NY..SRGAGAVS..DE.T.F.E. 1335
Zebrafish ...K..VL..V.VSVQL..S.NE.QQS..TQSV.-----.AAL.RDMR.T.L.SL.LTSS.Q..TS.VS..DE...SLE. 1309
Lamprey ..EIHP..AV.LSIS.T.RF.EE....NPE.EG..ES.NS.L.AD.......HLS-NGV.S..-DAVA..DAA..R.R. 1383
Human LTRERLGVNVTWKSRLEDIPVASLPDLHDIERALVGKDLLGRFTDLIQSGSFQLHLDSKTFPAE-TIRFLQGDHFGTSPR 1416
Xenopus .AVD........RTQ.D.F.ESFM.S.....D..I.DN.V...ASV.S..NYS.T....E.V.DRSVL.PHMEKL.SF.Q 1415
Zebrafish SNDKEVTARI.LMA..S.L.L.A.......DT..SEER..NGLKK..L...Y.-----------.-SI..SDHSLAL.SP 1376
Lamprey QDAL..L.....R.S.SRL..Q.Y....N....F.AH--AQ..AQ.LDDRGYR.A.NGRDYSS.GAA..RKDELYD...A 1461
2.1
1448
2.2
2.3
1.11
Human TWFGCSEGFYQVLTSEASQDGLGCVKCPEGSYSQDEECIPCPVGFYQEQAGSLACVPCPVGRTTISAGAFSQTHCVTDCQ 1496
Xenopus .SL..AQ..LKS.X.KTG.FN-...I..A..FA.N.V.KL..L....VEP.TAS.TK..L.T..VNG..YNRS...S.. 1494
Zebrafish PS.S.MT.YS.LPQ.T---------L..A..FFSGGV.T...RDTF..DE.QVF.S...A.TS.VAQ....AS..L.E.K 1450
Lamprey FRVA.EP.YVRIAQ-----------AA..R..LWR.GG.AL..ADH..DE..QT..T...ADTG.LT...AAAFQ.R.P.E 1533
Human RNEAGLQCDQNGQYRASQKDRGSGKAFCVDGEGRRLPWWETEAPLEDSQCLMMQKFEKVPESKVIFDANAPVAVRSKVPD 1576
Xenopus ..SI..L..A....QP....VATNRY....VF.K..I.S.ADTE.T.A...LLR...L...D.IL.IGEDSGVTQP.ANG 1573
Zebrafish KSK--.S.TTK.DFLSA..EPV...WR..SAQ.EE.T.TSSDGL.TVEE.KV.E...V.SS.SLLLKSESADTLS.DSSS 1528
Lamprey .EA--.---RR..LG.GERL----ELY..-.P.-----LY.G.K.AKGG.DEVRRM..E..SEDLVLG.VDA..L.IRTET 1599
3a.1
Human S--EFPVMQCLTDCTEDEACSFFTVSTTEPEISCDFYAWTSDNVACMTSDQKRDALGNSKATSFGSLRCQVKVRSHGQDS 1654
Xenopus Q..IKSLLD.I...GTE.S.DYIS...NGSGLI.EQ.SANES.JV.TDVQ.NESV...TDSVKIENPK..M.I.QRNTG. 1652
Zebrafish QPK.TQLRK.VL..AQ.DS.LYVA.FSD.EKTH.EM.STSA...E.R.TEPSKGF...DG.EA.QT.I.VL.IKGDEP.- 1609
Lamprey GPVQEVEPR..QE.LKE.R.D..VF..QGDVS..H..NGSRESYQ.EKAPEEKS...I.LCVV.IAIK.F.RGVGFHVS. 1679
3b.1
Human PA--VYLKKGQGSTTTLQKRFEPTGFQNMLSGLYNPIVFSASGANLTDAHLFCLLACDRDLCCDGFVLTQV--QGGAIIC 1730
Xenopus HT....G...YTFLRSG.AL..R...G.II..A.KTL....D..S.SE....HQV.RK.P.....I.S.IILNK.T.L. 1730
Zebrafish LT...LR...HEFS.AGL....RLS.RKAG..V.RTL..D.R.SA.A.V.R..VDS.G.ET.....I.N.NVLN..S.M. 1685
Lamprey ASGRF.FHA.HELPSWDGQLYR....G.AA..A.RTLALP.GTTT.P....Y..RS..SAQA.....L.KEL..PP.VLV. 1757
Human GLLSSPSVLLCNVKDWMDPSEAWANATCP-GVTYDQESHQVILRLGDQEFIKSLTPL------------------EGTQD 1782
Xenopus ......D......N..SGT.LLGGDGV.K...RSNK.QKMFSFF..G...TG.YSM.SESIGKVEYTTELTAEVK.EI.Q 1809
Zebrafish .F.TA.T..Q.SES..DVK.LSSSSRI.GA..Q.SKQLKRFTFSF.G.N.TITDAA.PAS...........SKNKT.Y.E 1754
Lamprey A...G.T..T.RAPW.PRTADEYGDGE.S..LRVHKP.RTFGFS..GVHYNA.NP.S..............AVASTAAA 1821
Human TFTNFQQVYLWKDSDMGSRPE-SMGCRKDTVPRPASPTEAGLTTELFSPVDLNQVIVNGNQSLSSQKHWLFKHLFS-AQQ 1869
Xenopus L.VT..R.F.KR.-----.GNG.LSD.SAGS.QELNNSSISDSVLD..L..ES.T.TTDPHLII...QYGVSRLRY..SD. 1884
Zebrafish .LIS..R.....E...NT.AKTLSA.SGSA.DEDSRSALSDSVK.A.DVLEGGD.N.DPERE.PN.LY.I...QY..F.E 1833
Lamprey AAAAS.VI...AG.EA...D-.VCLHT.AYT.Q.GE.AG.ADVSGR..TLRPEE.TLQPEMRVAQ.NF...RRA..PP.. 1899

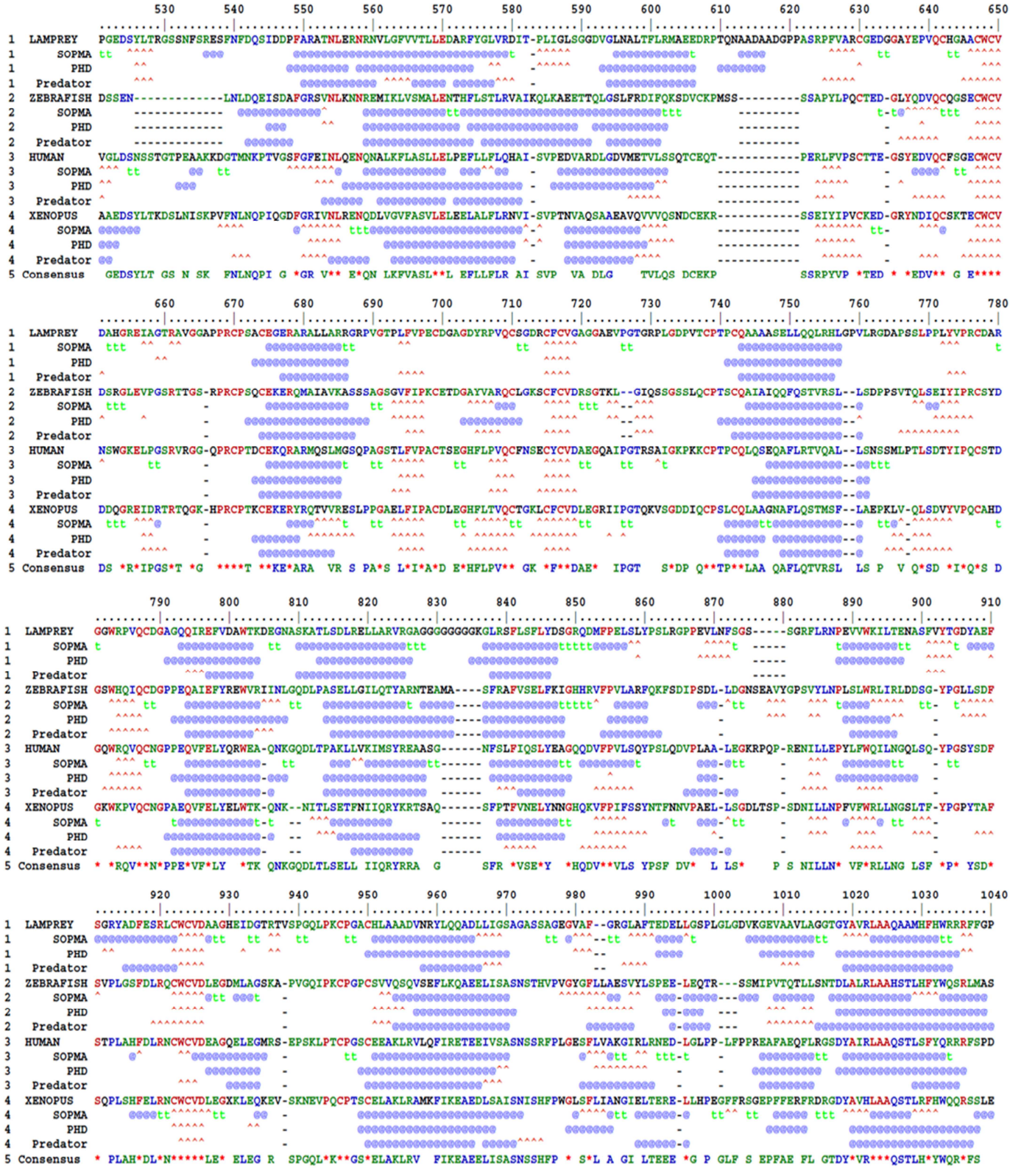


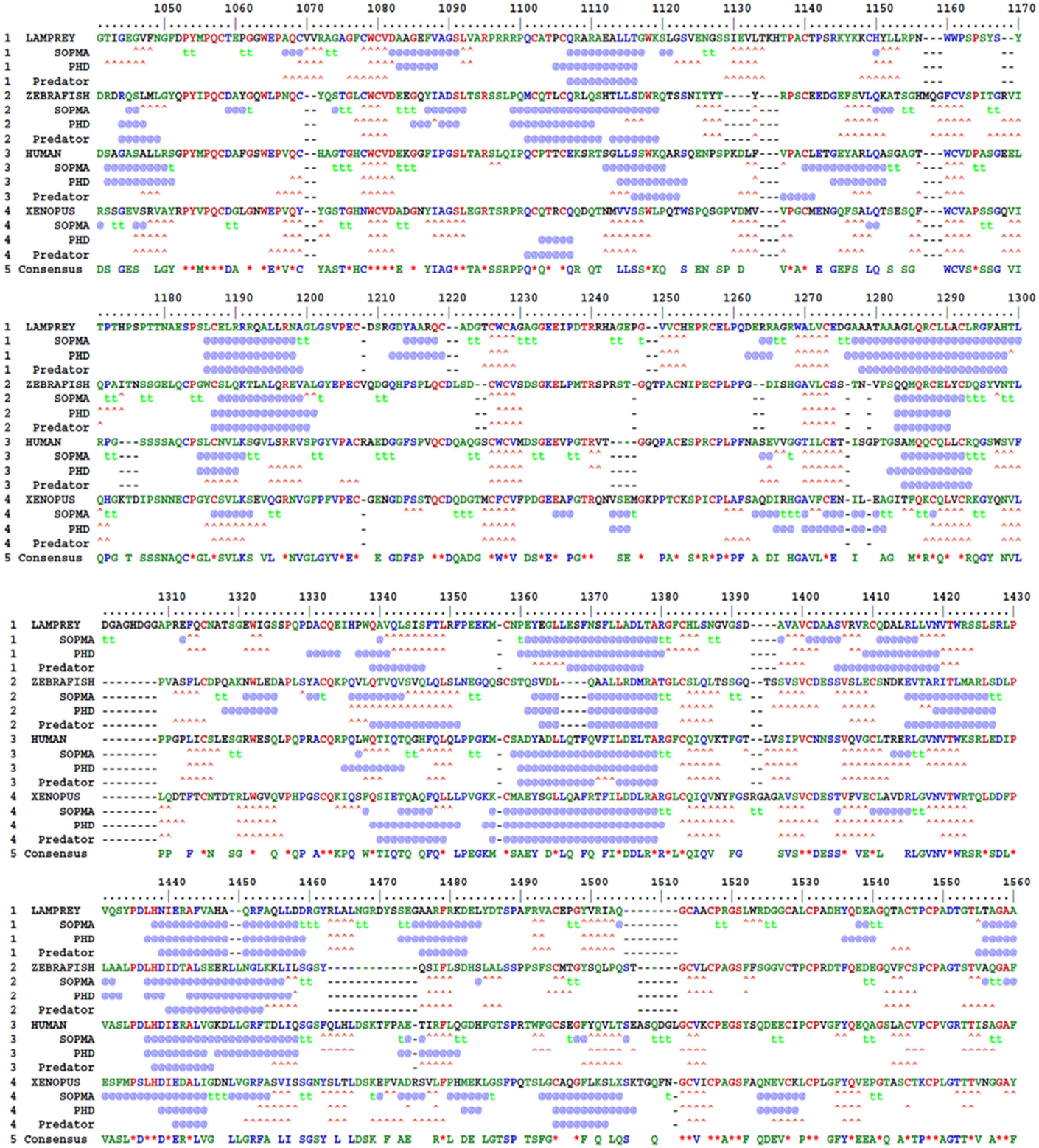
Supplementary Figure 1: Amino acid alignment of human, xenopus, zebrafish and lamprey Tg. The alignment was performed by MUSCLE. Tg1 repeat are highlighted in light blue, Tg2 repeat in red, Tg3 repeat in green and ChEL domain in grey. The donor tyrosines are highlighted in blue and the acceptor tyrosines in red.The conserved cysteins are highlighted in yellow. The 5'-end of Xenopus retrieve following mass sequencing is underlined in orange. The RNA probe for in situ are underlined in purple.

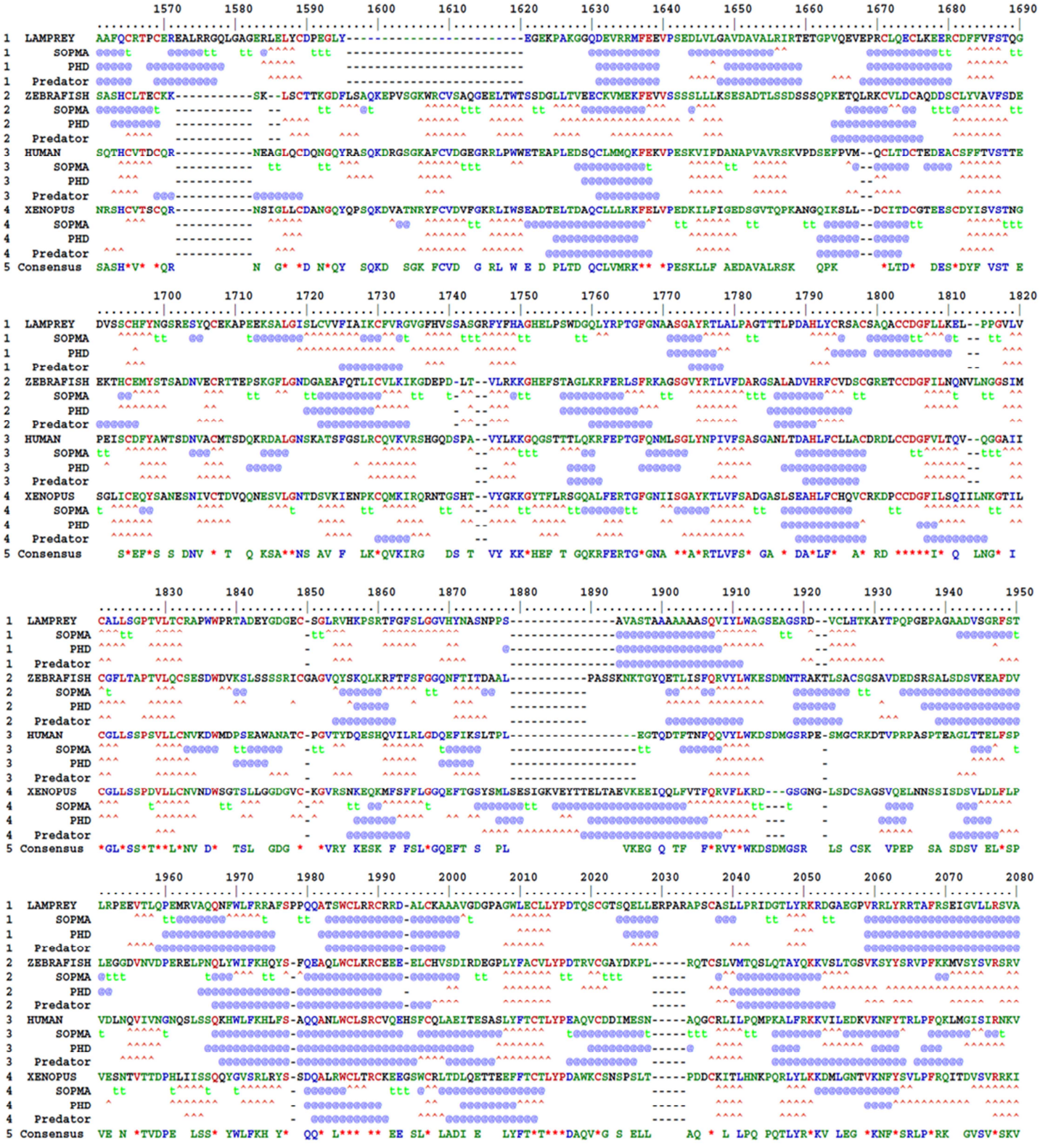


Supplementary Figure 2: Phylogenetic tree of amino acid sequences build by maximum likelihood (A)Tg1 repeat domain (B) Tg2 repeat and GCC2 GCC3 domain. SH-like branch support were computed and indicated in the tree when >0.70. Human, mouse, xenopus, zebrafish and lamprey sequences were used for vertebrate Tg sequences.

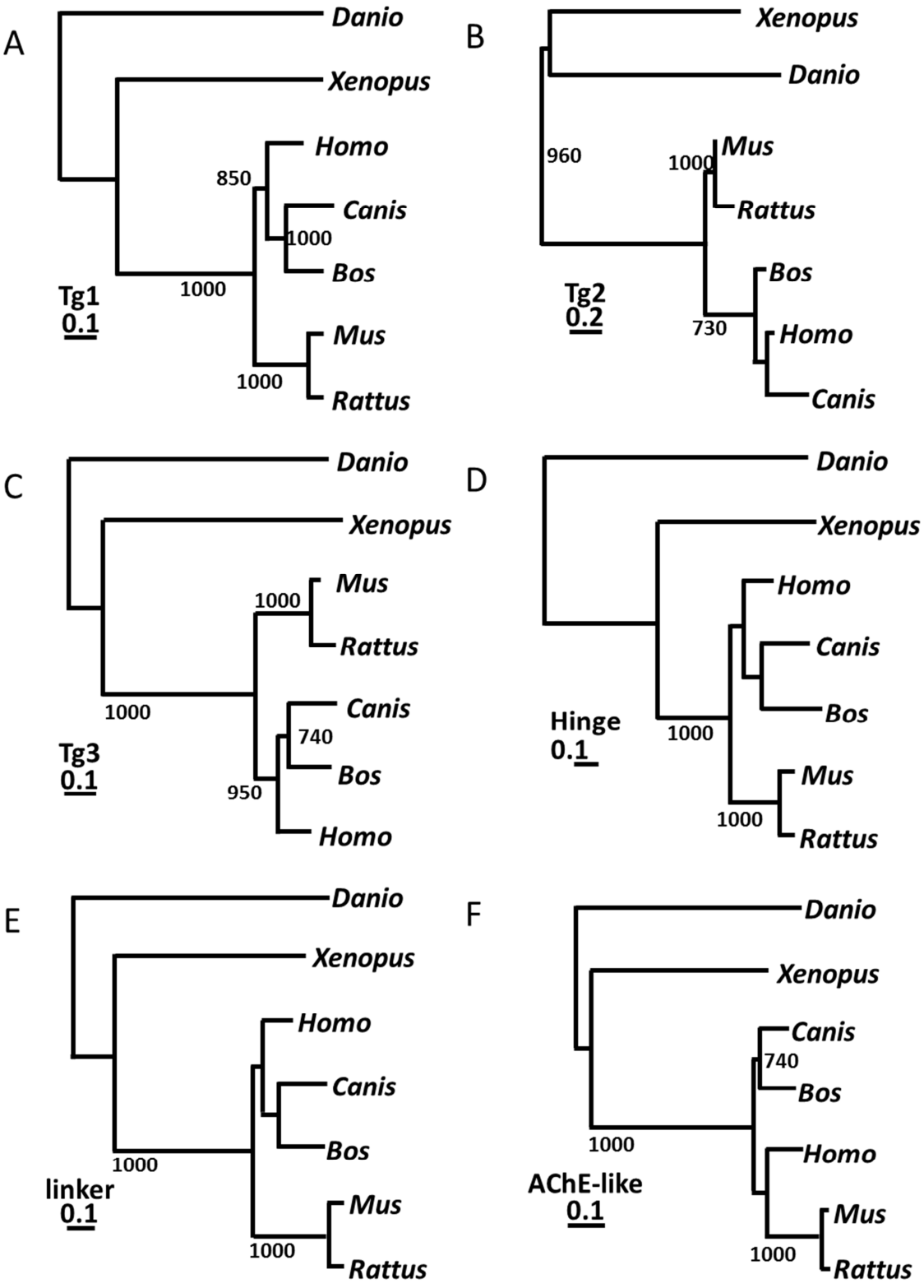





1 LAMPREY
1 SOPMA
1 PHD
1 Predator
2 ZEBRAFISH
2 SOPMA
2 PHD
2 Predator
3 HUMAN
3 SOPMA
3 PHD
3 Predator
4 XENOPUS
4 SOPMA
4 PHD
4 Predator
5 Consensus


1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680 1690
1 LAMPREY AAFQCRTPCEREALRRGQLGAGERLELYCDPEGLY----------EGEKPAKGGQDEVRRMFEEVPSEDLVLGAVDAVALRIRTETGPVQEVEPRCLQECLKEERCDFFVFSTQG
1 SOPMA
1 PHD
1 Predator
2 ZEBRAFISH SASHCLTECKK----------SK--LSCTTKGDFLSAQKEPVSGKWRCVSAQGEELTWTSSDGLLTVEECKVMEKFEVVSSSSLLLKSESADTLSSDSSSQPKETQLRKCVLDCAQDDSCLYVAVFSDE
2 SOPMA
2 PHD
2 Predator
3 HUMAN SQTHCVTDCQR----------NEAGLQCDQNGQYRASQKDRGSGKAFCVDGEGRRLPWWETEAPLEDSQCLMMQKFEKVPESKVIFDANAPVAVRSKVPDSEFPVM--QCLTDCTEDEACSFFTVSTTE
3 SOPMA
3 PHD
3 Predator
4 XENOPUS NRSHCVTSCQR----------NSIGLLCDANGQYQPSQKDVATNRYFCVDVFGKRLIWSEADTELTDAQCLLLRKFELVPEDKILFIGEDSGVTQPKANGQIKSLL--DCITDCGTEESCDYISVSTNG
4 SOPMA
4 PHD
4 Predator
5 Consensus SASH*V* *QR N G* *D N*QY SQKD SGK FCVD G RL W E D PLTD QCLVMRK** *PESKLLF AEDAVALRSK QPK *LTD* DES*DYF VST E
1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800 1810 1820
1 LAMPREY DVSSCHFYNGSRESYQCEKAPEEKSALGISLCVVFIAIKCFVRGVGFHVSSASGRFYFHAGHELPSWDGQLYRPTGFGNAASGAYRTLALPAGTTTLPDAHLYCRSACSAQACCDGFLLKEL--PPGVLV
1 SOPMA
1 PHD
1 Predator
2 ZEBRAFISH EKTHCEMYSTSADNVECRTTEPSKGFLGNDGAEAFQTLICVLKIKGDEPD-LT--VLRKKGHEFSTAGLKRFERLSFRKAGSGVYRTLVFDARGSALADVHRFCVDSCGRETCCDGFILNQNVLNGGSIM
2 SOPMA
2 PHD
2 Predator
3 HUMAN PEISCDFYAWTSDNVACMTSDQKRDALGNSKATSFGSLRCQVKVRSHGQDSPA--VYLKKGQGSTTTLQKRFEPTGFQNMLSGLYNPIVFSASGANLTDAHLFCLLACDRDLCCDGFVLTQV--QGGAII
3 SOPMA
3 PHD
3 Predator
4 XENOPUS SGLICEQYSANESNIVCTDVQQNESVLGNTDSVKIENPKCQMKIRQRNTGSHT--VYGKKGYTFLRSGQALFERTGFGNIISGAYKTLVFSADGASLSEAHLFCHQVCRKDPCCDGFILSQIILNKGTIL
4 SOPMA
4 PHD
4 Predator
5 Consensus S*EF*S S DNV * T Q KSA**NS AV F LK*QVKIRG DS T VY KK*HEF T GQKRFERTG*GNA **A*RTLVFS* GA * DA*LF* A* RD *****I* Q LNG* I
1830 1840 1850 1860 1870 1880 1890 1900 1910 1920 1930 1940 1950
1 LAMPREY CALLSGPTVLTCRAPWPRTADEYGDGEC-SGLRVHKPSRTFGFSLGGVHYNASNPPS----------AVASTAAAAAAASQVIYLWAGSEAGSRD--VCLHTKAYTPQPGEPAGAADVSGRFST
1 SOPMA
1 PHD
1 Predator
2 ZEBRAFISH CGFLTAPTVLQCSESDWDVKSLSSSSRICGAGVQYSKQLKRFTFSFGGQNFTITDAAL----------PASSKNKTGYQETLISFQRVYLWKESDMNTRAKTLSACSGSAVDEDSRSALSDSVKEAFDV
2 SOPMA
2 PHD
2 Predator
3 HUMAN CGLLSSPSVLLCNVKDWMDPSEAWANATC-PGVTYDQESHQVILRLGDQEFIKSLTPL----------EGTQDTFTNFQQVYLWKDSDMGSRPE-SMGCRKDTVPRPASPTEAGLTTELFSP
3 SOPMA
3 PHD
3 Predator
4 XENOPUS CGLLSSPDVLLCNVNDWSGTSLLGGDGVC-KGVRSNKEQKMFSFFLGGQEFTGSYSMLSESIGKVEYTTELTAEVKEEIQQLFVTFQRVFLKRD---GSGNG-LSDCSAGSVQELNNSSISDSVLDLFLP
4 SOPMA
4 PHD
4 Predator
5 Consensus *GL*SS*T**L*NV D* TSL GDG * *VRY KESK F FSL*GQEFT S PL VKEG Q TF F*RVY*WKDSDMGSR LS CSK VPEP SA SDSV EL*SP
1960 1970 1980 1990 2000 2010 2020 2030 2040 2050 2060 2070 2080
1 LAMPREY LRPEEVTLQPEMRVAQQNFWLFRRAFSPPQQATSWCLRRCRRD-ALCKAAAVGDGPAGWLECLLYPDTQSCGTSQELLERPARAPSCASLLPRIDGTLYRKRDGAEGPVRRLYRRTAFRSEIGVLLRSVA
1 SOPMA
1 PHD
1 Predator
2 ZEBRAFISH LEGGDVNVDPERELPNQLYWIFKHQYS-FQEAQLWCLKRCEEE-ELCHVSDIRDEGPLYFACVLYPDTRVCGAYDKPL-----RQTCSLVMTQSLQTAYQKKVSLTGSVKSYISRVPFKKMVSYSVRSRV
2 SOPMA
2 PHD
2 Predator
3 HUMAN VDLNQVIVNGNQSLSSQKHWLFKHLFS-AQQANLWCLSRCVQEHSFCQLAEITESASLYFTCTLYPEAQVCDDIMESN-----AQGCRLILPQMPKALFRKKVILEDKVKNFYTRLPFQKLMGISIRNKV
3 SOPMA
3 PHD
3 Predator
4 XENOPUS VESNTVTTDPHLIISSQQYGVSRLRYS-SDQALRWCLTRCKEEGSWCRLTDLQETTEEFFTCTLYPDAWKCSNSPSLT-----PDDCKITLHNKPQRLYLKKDMLGNTVKNFYSVLPFRQITDVSVRRKI
4 SOPMA
4 PHD
4 Predator
5 Consensus VE N *TVDPE LSS* YWLFKH Y* QQ* L*** ** EE SL* LADI E LYFT*T***DAQV*G S ELL AQ * L LPQ PQTLYR*KV LEG *KNF*SRLP*RK GVSV*SKV

Supplementary Figure 3: Alignment and secondary structure prediction of the human, xenopus, zebrafish and lamprey Tgs. The alignment we performed with MUSCLE and the prediction with the SOPMA, PHD and Predator softwares. @ indicates α-helixes, ^β-sheets and t turns in the sequences. The consensus sequences show * when an amino acid is shared by the four sequences, the amino acid letter when there is a majority consensus and no letter when there is no consensus.



Supplementary Figure 4:

Phylogenetic tree of amino acid sequences build by maximum likelihood with the cloned sequences of Tg on the several domains of the protein; A:Tg1, B: Tg2, C:Tg3, D: Hinge, E: Linker and F: ChEL-domain. 1000 bootstrap replicates were computed for the branch support and are indicated when > 1000.

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III. Discussion

In this thesis, we investigate the origin of TH signalling and its implication in metamorphosis through two axes. On the first axis tackle the question of the coordination of metamorphosis and ecological event with the coral reef fish *Acanthus triostegus*. We show that it undergo a TH controlled metamorphosis when it colonizes the reef. On the second axis, we investigate the origin of the TH signalling. We asked two questions, the origin of the signalling itself and the origin of the hormone. We use the annelid *Platynereis dumerilii* address if the TH/TR was functional. We show that is functional and involved in a developmental step that can be seen as the beginning of a metamorphosis. We investigate the evolution of the Tg, the central hormone of TH synthesis in vertebrates to understand the origin of TH synthesis. We show that Tg is a vertebrate novelty, raising the question of how TH is synthetized in non-vertebrates.

1. TH signalling is an old signalling

Before this work, the only group metazoan in which TH signalling was demonstrated as functional was the chordates (Laudet, 2011; Paris et al., 2008). There is evidence of TH related genes and metabolism of the hormone in tunicates and echinoderms, but the formal identification of a ligand binding on the TRs and the demonstration of the TRs ability to transactivate genes is still lacking. Therefore, the TH signalling is not proven as functional. There is no TR in ecdysozoans which rules out powerful model such as *Caenorhabditis elegans* and *Drosophila melanogaster* to investigate TH signalling. In lophotrochozoans however, genomic data show that TR and other genes of the TH signalling pathway are present. But identifying an ortholog *in silico* does not tell us its functions or properties. For instance, the nuclear receptor LXR and FXR bind bile acid derivative in vertebrate while EcR, their ecdysozoan ortholog binds ecdysone (King-Jones and Thummel, 2005). Therefore, bench experiments were required to investigate TH signalling at the scale of the bilaterians.

1.1. TH pathway was functional in urbilateria…

1.1.1. *Platynereis* data show a functional TH signalling

Our Investigation on *P. dumerilii* revealed that TH signalling is operational. The TR is expressed in the worm and it has the DNA binding, ligand binding and transactivation properties of a genuine TR. *P. dumerilii* TR binds the TRE as a vertebrate TR. It also binds several TH derivatives and transactivates a reporter gene in their presence. As there is a functional TR in chordates it is parsimonious to assume that TH signalling was functional in urbilateria, the last common ancestor of those two taxa (Figure 24).

Interestingly, Triac induces a transactivation response at a lower concentration than T3. Consistently, higher dose of NH3 (a competitive antagonist Lim et al., 2002) are required to inhibit

Triac induce transactivation than T3 induced transactivation. Together, it means that, in our transient transfection system, Triac has a stronger activity on the TR than T3. This is interesting given that Triac is the identified agonist of the Amphioxus TR (Laudet, 2011; Paris et al., 2008a). In other words, the most basal TR proven has functional before our study, has Triac and not T3 as a ligand. Therefore it is relevant to consider the hypothesis of Triac as a potential ancestral TR ligand and not only T4 or T3.

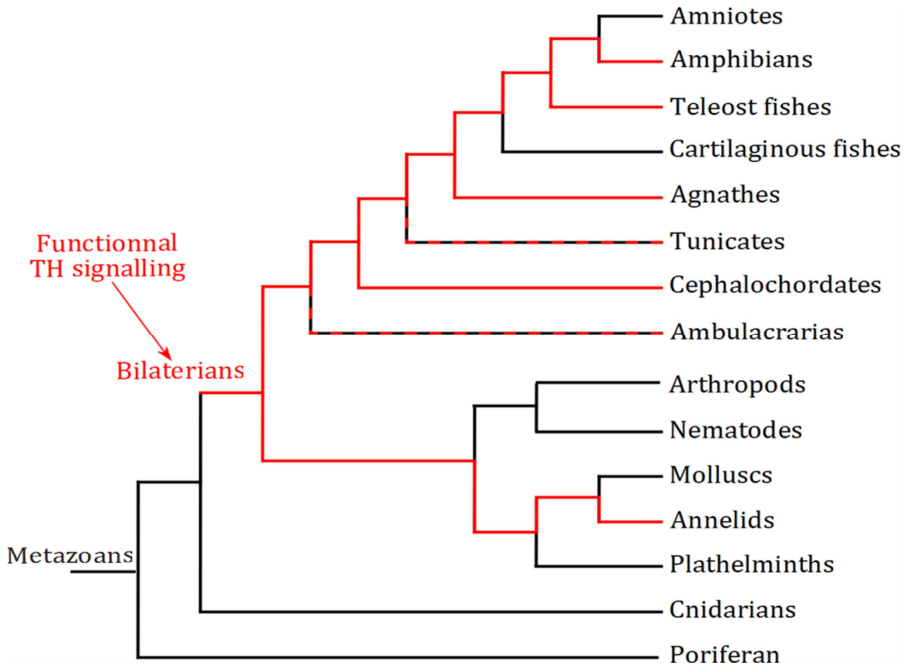

Figure 22: Simplified tree of metazoans. Our findings in *P. dumerilii* push back the origin of TH signalling at the basis of bilaterian.

1.1.2. Genomic data from other annelids and molluscs

As we were interested only in one species, one could still argue that the TH signalling observed in *P. dumerilii* is not homologous the chordate TH signalling but an unlucky convergence. Meaning that the TH signalling appeared independently in chordate and *P. dumerilii.* Thus, it worth investigate more species to better understand the situation in urbilateria.

The annelid *Capitella telata* and the mollusk *Aplysia californica* are interesting organisms to complete our knowledge of TH signalling in lophotrochozoans .Their genome is sequenced and they are used as experimental models (Moroz et al., 2006; Simakov et al., 2012). Available data show that these organisms have a TR as well as other genes of the TH signalling pathway. A first glimpse of an answer is given by *A. californica.* This sea slug harbours a peroxidase gene that is ortholog to the vertebrate TPO. Experiment performed with the iodine isotope I^{125} show that the sea slug was able to incorporate it to T4, indicating an endogenous synthesis of TH (Heyland et al., 2006). Therefore it brings another lophotrochozoan with a putative TH metabolism. This gives an orientation to find other functional TR in lophotrochozoans. It is important to emphasize on the fact that finding a

functional receptor, and not only the hormone, is mandatory to assess the TH pathway as functionnal in another group.

1.1.3. Outside bilaterian

The genomic data of the cnidarian *Nematostella vectensis* (Putnam et al., 2007) or the porifera *Amphimedon queenslandica* (Srivastava et al., 2010) show no clear ortholog of TR. Some targeted cloning of TR in the cnidarian *Acropora millepora* fail the find any ortholog (Grasso et al., 2001) and the general consensus is in favour of an origin of the TR in urbilateria (Tarrant, 2005).

1.2. ...but TH synthesis was most probably different

1.2.1. No Tg nor Thyroid outside vertebrates

In our work, we conclude that Tg is a vertebrate novelty. However, it is well established that TH signalling is functional in the amphioxus (Paris et al., 2008b; Wang et al., 2008) and our study *P. dumerilii* shows a functional TR. Therefore, is TH synthetized in non-vertebrate organism? If yes, how is it done since there is neither Tg, nor thyroid? Two hypotheses can be drawn (i) TH is endogenously synthetize, in a different manner than in vertebrates. (ii) TH has an exogenous origin.

1.2.2. Hypotheses for the origin of TH synthesis

1.2.2.1. Endogenous synthesis

The endogenous hypothesis states that non-vertebrate metazoans are able to synthesise TH without thyroid and Tg. Since TH is made by the conjugation of two tyrosines (Gavaret et al., 1981), any other protein than Tg could be the backbone of this reaction under this hypothesis. Interestingly, there is a deactivating deiodinase in amphioxus (Klootwijk et al., 2011) and a putative deiodinase in ascidian (Sheperdley et al., 2004), indicating that TH level might be tightly regulated Moreover, there putative orthologs of TPO in echinoderms and molluscs (Heyland et al., 2006; Miller and Heyland, 2013) with evidence of a TH synthesis.

1.2.2.2. Specific iodination of the endostyle

The endostyle of non-vertebrate metazoans could be the place of such a synthesis. The endostyle of deuterostome is homologous to the thyroid gland. This is supported by observation of lamprey development, in which the larval endostyle becomes the adult thyroid (Kluge et al., 2004). Moreover the genes *nk2.1/ttf-1*, *pax8* or *foxE*, that are critical for the thyroid development of vertebrates are expressed in the lamprey and amphioxus endostyle (Hiruta et al., 2005; Mazet, 2002). In protostomes, however, the homology of the foregut with the deuterostomes endostyle is not certain. Indeed, in deuterostome, the endostyle derived from endoderm whereas the protostome foregut derived from the ectoderm. Nevertheless, some cell in the foregut of *P. dumerilii* expresses of *nk2.1*

(Christodoulou et al., 2010) and a *fox* ortholog is expressed in the foregut of *C. teleta* (Shimeld et al., 2010) .

One interesting feature of the endostyle is the accumulation of iodine that partially overlaps with the expression of TPO in *Ciona intestinalis* (Barrington and Franchi, 1956; Hiruta et al., 2005) and amphioxus (Barrington, 1958; Hiruta et al., 2005). Interestingly, an accumulation of iodine in *P. dumerilii* has been described in the pharynx (Gorbman, 1955), where iodine has the property to stiffen the mucus and enhance food trapping.

1.2.2.3. Other similarities between the endostyle and the thyroid

Aside from the molecular signature and the accumulation of iodine in the thyroid of vertebrates, endostyle of deuterostomes and foregut of protostomes, there are some interesting structural and physico-chemical properties that are shared by those three structures. The non-vertebrate endostyle/foregut ensures a mucus secretion in order to trap food particles. Similarly, the thyroid, consists in thyrocytes enclosing the colloid a thick gel made of glycoprotein (Figure 5; van de Graaf et al., 2001) . While the thyroid accumulates iodine, in an extracellular compartment to ensure TH synthesis, the endostyle/foregut accumulates iodine, which is known to increase viscosity (Ueda et al., 1998), to enhance food trapping. Therefore the same phenomenon of iodine accumulation happens on those structures irrespectively of their function.

As explained before, TPO orthologs of *C. intestinalis* and the amphioxus are expressed in their endostyle. Interestingly all the vertebrate peroxidases, are excreted and perform their activities in a glycoprotein environment or a mucus. TPO is addressed to the apical membrane of the thyrocyte (van de Graaf et al., 2001). The eosinophil peroxidase is found the vacuoles of the eosinophils, which are acidic mucus (Egesten et al., 1986). The Peroxidasin, Lactoperoxidase and Myeloperoxdase are found addressed toward diverse mucus, granules and extracellular matrices (Egesten et al., 1994; Hagen et al., 1976; Péterfi et al., 2009). Thus all the peroxidase, including TPO, functions under similar physical and chemical parameters irrespectively of their function.

Another similarity between the thyroid, the deuterostome endostyle and the protostome foregut lies in the protein they secrete. The thyroid secretes the Tg in the colloid. Evidence suggest that the deuterostome endostyle secretes proteins of the mucin family (Barrington and Franchi, 1956; Hosp et al., 2012) .There are also good evidence of the presence of mucin in the protostome foregut. There are mucin orthologs in the genome of *P. Dumerilii* (Trachana, 2011) and biochemical evidence of mucin-like protein in the foregut of *Nereis virens* (Michel and DeVillez, 1980). Both proteins are large, 2700 amino acid for the Tg (Malthiéry and Lissitzky, 1987) and up to 6000 amino acid for the mucin (Dekker et al., 2002) . Both proteins are evolutionary unrelated but have similar structural feature. They both undergo an important post-translational modification during their maturation (Dekker et al., 2002; van de Graaf et al., 2001). Both protein harbour many cysteins that form disulfide bound critical for their 3D folding. Both proteins are made repeated functional domains, the Tg repeat for the Tg and the Von Willebrand and MUC domain for the mucins. Moreover, both proteins have predicted hydrophobic pocket that are important for protein-protein interaction or the docking of another compound (Dekker et al., 2002). Could mucin be the backbone of endogenous TH synthesis outside vertebrates?

To summary, the genetic fingerprints support a homology of the thyroid with the deuterostome endostyle. This homology is dubious for the protostome foregut. Strikingly, those structures allow the formation of a local environment with some shared physico-chemical properties: there is accumulation of iodine in an extracellular mucus, there are peroxidase and large proteins secreted. This makes the endostyle a good candidate for the endogenous synthesis of TH in non-vertebrate metazoans.

1.2.3. Food origin

The alternate hypothesis is the external origin of the TH, from food. This is supported by the evidence of iodinated compound in algae, including T4 (Heyland and Moroz, 2005; Miller and Heyland, 2010) . Some authors suggest that the algae could be involved in the metamorphosis of ambulacrarias by providing TH (Chino et al., 1994; Heyland et al., 2004) . Under this hypothesis, TR would have a sensor activity, indicating the food availability (Miller and Heyland, 2010). Given that metamorphosis is a demanding and energy consuming event (Heyland et al., 2004; Wright et al., 2011) TR bound with food derivative compounds would give the signal to undergo metamorphosis. Latter, in vertebrate common ancestor, the endostyle would have been internalized as a thyroid gland. The TH synthesis would switch from external to internal, maybe to allow a more precise adjustment of the TH signalling . This would not be unique case of a cross-talk between plant compounds and metazoan nuclear receptor. This would not be a unique case among nuclear receptor since some plants defend themselves against herbivorous insects by producing compounds that disturb their fatty-acid and steroid signalling (Schultz and Appel, 2004).

As a conclusion, there are arguments supporting the two hypotheses. However, data are scarce on this subject and limited to a small range of species. There is no enough evidence to build a robust framework to understand the evolution of TH synthesis. Thus, *P. dumerilii*, as a distant relative of deuterostomes is an interesting species to investigate to understand both the TH signalling and the TH synthesis. Any finding in the worm is one more step toward the understanding of the evolution of the TH pathway.

2. Toward an evolutionary definition of metamorphosis?

2.1. Metamorphosis as an environmentally integrated event in many taxa

Metamorphosis is not only a purely developmental event since larvae and juveniles of a given species often have different ecological niches. Investigation from amphibian shows a role of the environment through the stress axis and CRF signalling in the control of metamorphosis (Denver, 2009a) . The role of the environment in the triggering of metamorphosis in other species, however, is less clear. There are a many studies on salmonids demonstrating a clear link between day lengthening, HPT axis and smoltification (Björnsson et al., 2011). Nevertheless, data from other teleosts are scarce and there is no general framework to understand the role of environment in teleosts metamorphosis and how this event integrates in the fish ecology.

This is the context of our investigation on coral reef fishes. We show that TH is involved in the metamorphosis of the convicted surgeonfish *A. triostegus*, and that the hormonal shift and the ecological event of the reef colonization happen are the same time. The hormonal dosages of *Rihnecantus aculeatus* and *Chromis viridis* go in the same direction with a drop of TH level around the reef colonization. From this we can hypothesize that this is a general scheme of coral reef fish: the TH-controlled metamorphosis and the colonization are the same event. From this we can build a first hypothesis that is, the more a fish colonize the reef with a juvenile morphology (*e.g. R. avuleatus*), the more the TH shift happens early in the approach of the reef. On the contrary, species that morphologically turn into juvenile right after entering the reef (*e.g. A. acanthurus*) would have a later TH shift. Therefore, the change in TH signalling may impact the phenotypic manifestation of the metamorphosis, in terms of timing and magnitude and its integration with environmental and ecological factors. Thus, difference in metamorphose timing and magnitude could be involved in difference of history life trait.

This opens the question of the coordination of metamorphosis with the environment. Indeed, larvae of coral reef fishes develop in the open ocean and all of them do not have the same absolute age when they colonize the reef (McCormick, 1999). Moreover, it is known that coral reef fish larvae actively swim toward the reef (Fisher, 2005), following acoustic and olfactive clues (Lecchini et al., 2005; Parmentier et al., 2015) during reef colonization. Thus, it gives a hint to understand how development can be coordinated with the ecology (*i.e*. the geographical position of the fish in respect of the reef). Acoustic and olfactive clues could trigger the HPT axis. This can be direct as exemplified by the day lengthening response of salmonids (Lorgen *et al.*, 2015), or indirect through the HPA axis as exemplified as the stress-induced metamorphosis of amphibians (Denver, 2009a) . The role of the HPA axis should be considered given that GC influence the embryogenesis of the damselfish *Pomacentrus amboinensis*, indicating that they have a biological role in coral reef fishes (Mccormick & Nechaev, 2002). This is the working hypothesis we propose to understand the integration of development and ecology of the coral reef fishes.

The role of the environment in metamorphosis is interesting to put in perspective with a recent finding in *P. dumerilii.* In this annelid, the settlement, which corresponds to the end of the troochophore stage and often referred as the settlement metamorphosis, can be triggered by the secretion of the neuropeptide MIP (Conzelmann et al., 2013). Interestingly, this neuropeptide is secreted by chemosensory neurons that project sensory dendrite to a permeable space of the cuticle (Conzelmann et al., 2013). Thus, the settlement is *P. dumerilii* is under the influence of environmental clues, although they are not identified yet. This would indicate that the influence of the environment on metamorphosis is an old feature.

2.2. When did the TH control of metamorphosis appear?

The expression profile of TR in *P.dumerilii* is particularly interesting as a peak of expression preceded the settlement metamorphosis. Accordingly, T3 and Triac treatment performed between 6 and 18 hpf accelerates the development of the pre-settling larva at 48 hpf but does not affect the settling itself. In other words, the TR is expressed when the larvae begins its trochophore to nectochaete transition and exogenous T3 and Triac accelerates this transition but not the settling of the larva. This would indicate a role of TH in the development of *P. dumerilii* and maybe in the initiation of the settlement metamorphosis. In other annelid species, such as the genus *Malacoceros*

the trochophore step is much longer, in another species *Sabellaria cementarium*, the larva feed during this stage (Pernet and Strathmann, 2011). This is not the case of *P. dumerilii* (Fischer et al., 2010). Thus, *P. dumerilii* could be an example of fast developing annelid. This would explains why the TH treatment works in a small time window: if the development is already fast, it is complicated to overly accelerate it.

Given the expression profile of TR and the outcome of the pharmacological treatment, we propose that TH is involved in the control of the settlement metamorphosis of *P. dumerilii*. As a consequence, it suggests a role of TH in metamorphosis in urbilateria. Nevertheless, as for the origin of TH signalling, more investigation, preferentially in other taxa than annelid, is required to assess to confirm this hypothesis.

2.3. TH peak in non-metamorphosing taxa: amniotes

The following part has been adapted form 5.1.: Holzer, G. and Laudet, V., 2013, Thyroid Hormones and Postembryonic Development in Amniotes. Current Topics in Developmental Biology, Volume 103, p397-425.

To have a complete view on the role of TH in metazoan development, it worth to investigate what happens in taxa without a metamorphosis: the amniotes.

There are large disparities among mammals, between altricial or precocial development strategies and most of the data come from rat and mouse. Nevertheless, in the late development/early postembryonic life of mammals, it seems clear that a peak of TH is mandatory for the normal development of organs such as intestine or brain. This peak is reminiscent of the amphibian metamorphosis. In eutherians, this peak is concomitant with brain and intestine development (Fraichard et al., 1997; Plateroti et al., 2001). Moreover, this peak is apparently delayed after birth in the species, such as mouse (Hadj-Sahraoui et al., 2000) , requiring a long post-birth development until autonomy. In contrast, this peak can occur very early in species with long gestation time exhibiting precocial strategies: this is the case of the guinea pig (Abalo et al., 2009; Castro et al., 1986). In human, the peak is concomitant to birth (Erenberg et al., 1974). In metatherians, it is apparently concomitant with the establishment of endothermy, fur, intestine development, and the first leaving of the pouch, that is to say, the beginning of autonomy (Richardson et al., 2002; Setchell, 1974) .

In sauropsids, most of the data on TH level around hatching come from studies performed in birds (Olson et al., 1999; Wilson and McNabb, 1997) and the publication on heterotherm sauropsids are scarce (Richardson et al., 2005; Shepherdley et al., 2002) . However, taken together, these data suggest a different TH regulation between precocial and altricial birds with a near-hatch peak for the former and a smoother late one during posthatch development for the later. This fits with the notion of a TH peak related to autonomy of the hatchling, an idea that was proposed by McNabb several years ago (McNabb, 2006; McNabb et al., 1984). Indeed, precocial birds tend to have thermoregulatory independence and feed themselves, whereas altricial birds rely on their parent for heating and feeding. Moreover, precocial hatchling such as quail appears to be resistant to thyroid manipulation with goitrogens, indicating that (i) no further important maturation controlled by TH seems to occur in these birds after hatching and (ii) TH metabolism is already mature in these birds as the hatchling respond to goitrogen in a manner indistinguishable to the adults. As far as other sauropsids are concerned, indirect evidence indicates that a peak of TH could exist at hatching in

some species such as crocodiles, but few data are available. As we previously proposed for mammals, the TH peak at birth could be an indicator of the degree of independence of the hatchling in sauropsids with a peak of TH just before or at hatching correlating with an independent autonomous offspring. This acquisition of autonomy allowed by the TH trigger could be considered as being equivalent to the amphibian metamorphosis, although this is only a speculation given the data available.

Overall, what we observed in mammals and bird species for which data are available is a stage of organ maturation that corresponds to a period of autonomy acquisition by the young that is concomitant to a TH peak, and probably a period of high sensitivity to the hormone. We propose that this period should be considered as equivalent to Anuran metamorphosis, and defined as a TH-controlled functional remodeling of organs allowing the maturation of main physiological system preparing the entry into a new ecological niche.

2.4. The definition of metamorphosis

Earlier in this manuscript, we took definition of metamorphosis as the transition period between a larvae and a juvenile where the individual undergo spectacular physiological, behavioural and ecological modifications. If this definition suits well for the amphibian metamorphosis, it is not fully satisfying on the light of the metamorphosis in other animals.

2.4.1. Current definition what does “spectacular means”

The main problem with this definition is the use of the term spectacular which is subjective. The transition of a tadpole into a frog referred as spectacular since some whole body part grow (*e.g.* the limbs) and other disappear (*e.g.* the tail) in a few weeks (Brown and Cai, 2007). It also suits well for the definition of flatfish metamorphosis with a change of symmetry. On the contrary, the zebrafish undergo a cryptic metamorphosis that is much less spectacular than the flatfish one since it consist mainly in the establishment of the adult pigmentation and the fin growth (Brown, 1997; McMenamin *et al.*, 2014). Are these changes substantial enough to be referred as spectacular? Given that it is TH that controls metamorphosis in all teleost, it is the same event happening in both zebrafish and flatfish but with different strength.

2.4.2. The definition is not satisfying: it does not reveal biological historicity

The current definition of metamorphosis take into account developmental and ecological changes but it does not consider the evolution of this event. To assess the homology of a gene or of a structure, one must consider different species and investigate the historical continuity between those genes or structure (Wagner, 2007). This allows understanding how these genes or structure are related despite genetic drift, species peculiarities or extreme examples. Thus it should be the same for developmental event such as metamorphosis. This would allow establishing -or ruling out- the homology of developmental events and to go beyond the specificity of each species, such as heterochrony, or the cryptic character of some metamorphoses. This is exemplified by the direct developer *E. coqui* that undergo a metamorphosis within the egg, despite no visible tadpole stage (Callery & Elinson, 2000). This raise the question of the peak of TH observed in amniotes. It is likely that there is an historical continuity between this peak and the metamorphosis of amphibian. Should

the definition of metamorphosis integrate an evolutionary perspective? Should we define a developmental step controlled by endocrinological actor of which metamorphosis would be an extreme example?

3. At the scale of meatzoans: TH, Ec and ‚non-bilaterian metamorphosis

3.1. The ecdysone-controlled metamorphosis

So far, we only discussed about the TH controlled metamorphosis, but this is forgetting the other identified hormonal system that controls metamorphosis, the ecdysone (Ec) pathway (Thummel, 1996). Ec controls the moutling and metamorphosis of arthropods. We will consider here only the insect the metamorphosis since it is the best described.

Ec is derived from a steroid precursor and therefore originates from lipid metabolism. It binds the Ec receptor (EcR) which is a nuclear receptor (Laudet and Gronemeyer, 2001) and function as a heterodimer with USP (Yao et al., 1993), the ortholog of RXR. It is a peak of Ec that triggers insect metamorphosis through EcR signaling and activation of its target genes (Thummel, 1996). Ec release is under the control of central nervous system through a casacade of neuroendocrine factors (Smith and Rybczynski, 2012). There is some evidence that insect development is influenced by environmental factors, such as food availability (Shafiei et al., 2001).

3.2. Metamorphosis: different pathway the same pressures?

TH and Ec controlled metamorphosis are driven by two different hormones, which are not homologous and have huge differences. For instance, insects are sexually mature after their metamorphosis. Nevertheless, there are some similarities that could help us to pinpoint the selective pressures that might act upon metamorphosis.

3.2.1. Ecological transition: compete less, spread more

In both systems, the larvae often undergo an ecological transition that comes with physiological, morphological and behavioural changes. Thus, often, the larva and the adult do not occupy the same ecological niche and do not compete for the same food resources or habitat. The metamorphosis often allows a separation between dispersal and sedentary phases. Larvae can be sedentary and adult dispersal, as for amphibians and flies but it can be the other way around with dispersal larvae and sedentary adults as in many marine species (Hadfield, 2000). This dispersion would enhance the surviving of the species (Mayhew, 2007).

3.2.2. Pathways linked to metabolism

The other similarity is that in both cases it is an endocrinological signal going through a nuclear receptor that controls metamorphoses. Moreover those two pathways are involved in metabolism control in their respective taxa (Bonneton and Laudet, 2012; Mullur et al., 2014). This is not a

surprise given that: (i) metamorphosis is a developmental process and (ii) metamorphosis is demanding in term of energy. As examples, insect larvae and tadpole will consume most of their fat during their metamorphosis (Aguila et al., 2007; Wright et al., 2011). Thus it is conceivable that a pathway involved in the energy expenditure has been selected to be the trigger, or the checkpoint, of the metamorphosis.

3.3. The metamorphosis of cnidarians

Cnidarian have a life cycle with several steps. Cnidarian zygotes develop into planula larvae which settle to form polyps. Polyps undergo asexual clonal reproduction by budding and form a colony. Polyps also undergo a strobilation and turns into a medusa, except in the anthozoans taxa (*i.e.* corals and sea anemones) that sexually reproduce as polyps. The medusa reproduces sexually and starts the cycle again (Figure 25; Miglietta and Cunningham, 2012).

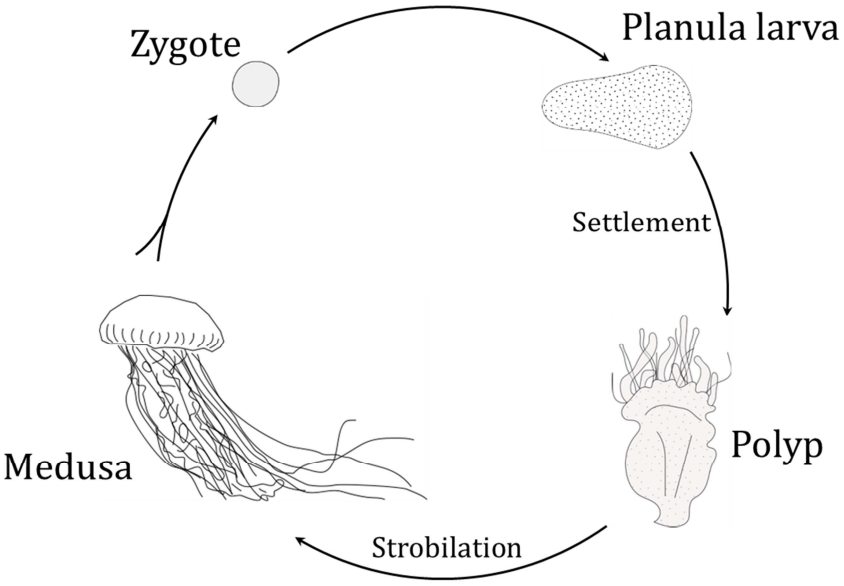

Figure 25:. Developmental cycle of Cnidarian.
Zygotes develop into planula larvae. The planula larvae settle into polyp where they can undergo asexual proliferation. Polyps can strobilate into medusa that reproduce sexually. Anthozoans do not have a medusa form and sexually reproduce as polyps.

Interestingly, in *Aurelia aurita* the RXR ortholog is involved into cnidarian strobilation (Fuchs et al., 2014). Strikingly, some studies suggested that TH and iodinated compounds can induce the strobilation of *Aurelia aurita* (Spangenberg, 1971; Spangenberg et al., 1994) despite the absence of identified TR ortholog. Thus, how this signal is integrated? Is it a direct effect or the result of the degradation of those compounds?

The settlement of *Acropora palmate* can be induced by a neuropeptide called the GWL-amide (Erwin and Szmant, 2010) . Interestingly, this GWL-amide peptide and the MIP inducing settlement in *P. dumerilii* belong to the same neuropeptide family (Conzelmann et al., 2013). Strikingly, MIP ortholog of insects modulate the Ec signalling (Hua et al., 1999).

This supports the idea for further investigation of the settlement and strobilation of cnidarians. Is it a lucky coincidence to find actors of the TH and Ec controlled metamorphosis in cnidarian or is it biologically relevant? Gathering more data on the mechanism that control and signal the major life transition in cnidarian can be of great help to understand the evolution of metamorphosis in metazoans.

IV. Conclusion

We cannot yet understand the full historicity of the metamorphosis at the scale of metazoan. Nevertheless, accumulating data in different species, from *Xenopus leavis* to *Aurelia aurita* draw the scheme a developmental event that integrate environmental clues through internal relay such as the endocrine signalling.

It is unclear to what extend those metamorphosis are related one to another. It is also unclear if there is a qualitative difference between what is defined as a metamorphosis of some species and growing in another. Those questions can be debated over and over and the reality is certainly more complicated than we think.

Progresses are made and our understanding of metamorphosis is getting better and better thanks to the integration of the evolutionary, developmental, ecological, physiological and genetic approaches. Understanding how variation on a developmental event such as metamorphosis can shape the wonderful biodiversity we enjoy today is at the very heart of evo-devo.

V. Appendix

1. Thyroid Hormones and Postembryonic Development in Amniotes

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