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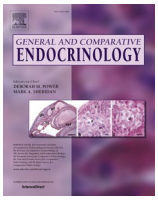

Metamorphosis-associated immune system maturation in Senegalese sole☆

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ABSTRACT

The thyroid hormones (THs) are proposed as putative regulators of immune system maturation in developing teleost fish. To gain insight into this process the Senegalese sole (*Solea senegalensis*) that has a well-characterized TH-driven metamorphosis was used. Differential gene expression analysis was performed across developmental stages (n = 3 per stage): pre-metamorphosis, onset of metamorphosis, metamorphosis, early climax, climax, and post-metamorphic juveniles. Metamorphosis is a massive gene-oriented developmental process, involving the differential expression of 8145 genes. Clustering analysis was used to identify immune-related genes with similar expression patterns to hypothalamus-pituitary-thyroid (HPT) axis-related genes. TH-regulated candidate immune genes were identified (133) and analysis of their promoter region revealed 84 contained putative TH receptor (TR) binding sites (TREs). Two consensus TRE sequences were identified in the candidate genes, 5′-*ntgn*GntCac*a*n (exclusive to TRα), and 5′-*nnn*tGgtC*a*n*nn* (common to both TRs). TRα-exclusive TREs were less common than those that bound interchangeably TRα and TRβ. In the promoter region, TRα-exclusive TREs were always accompanied by the pan-TRE consensus sequence, never occurring independently.

1. Introduction

In amphibians and fish, thyroid hormones (THs) are recognised for their pivotal role in the larval/juvenile transition (a.k.a., metamorphosis) and a suite of significant and irreversible developmental and physiological modifications that occur during this process (Alves et al., 2016; Laudet, 2011; Manchado et al., 2008, 2009; Shao et al., 2017; Shi et al., 1996; Tanaka et al., 1995; Tata, 2000; Wong and Shi, 1995; Yamano and Miwa, 1998). The absolute requirement for THs in metamorphosis is underscored by experiments showing that removal of THs prevents metamorphosis and reduces larval survival (Iziga et al., 2010; Manchado et al., 2008). THs exert their action by binding to nuclear thyroid hormone receptors (TRs) (Dai et al., 1996) that in turn activate expression of TH-responsive genes (Bizhanova and Kopp, 2009; Dai et al., 1996; Moreno et al., 2002; Ohye and Sugawara, 2010). These responsive genes contain thyroid response elements (TREs) in the promoter region, which in mammals are typically conserved as hexameric (G/A)GGT(C/G)A (Cheng, 2000) or octameric TAAGGTCA (Katz and Koenig, 1994) sequences. However, the primary nucleotide sequences of TREs including the number, spacing, and orientation of the half-sites can vary (Yen, 2001). In teleost fish, the specific mechanism of TRs' action and the TRE half-site sequences are not fully understood but are assumed to be similar to those of other vertebrates (Almuly et al., 2000; Isorna et al., 2009; Oku et al., 2002; Sternberg and Moav, 1999).

Metamorphosis is a critical developmental stage during which organogenesis and maturation of various physiological systems, including the digestive, cardiovascular, and central nervous systems occurs (Gomes et al., 2015; Najafpour et al., 2024; Palacios-Martínez et al., 2020). Much of our understanding of fish metamorphosis comes from studies in flatfish (Pleuronectiforms), which undergo a dramatic transformation from a bilaterally symmetric larvae to an asymmetric juvenile flattened laterally along the dorsoventral plane (Darras et al., 2015; Geffen et al., 2007; Power et al., 2001; Power et al., 2008; Sæle et al., 2006; Schreiber, 2013). Hence, flatfish has become a valuable biological model for studying metamorphosis due to their clear and identifiable developmental changes. During metamorphosis, one of the eyes moves across the dorsal midline to the opposite side of the head, and this change allows easy tracking of metamorphic progression. Eye

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migration is linked to other significant morphological changes such as head remodelling, alterations in pigmentation, and the loss of the swim bladder, events that are closely associated with a peak of THs at the metamorphic climax (Klaren et al., 2008; Manchado et al., 2008, 2009; McMenamin and Parichy, 2013). These notable and well-defined characteristics during metamorphosis and their dependence on THs makes flatfish an ideal model system to study TH-regulated post embryonic development (Benzekri et al., 2014; Dinis et al., 1999; Fernández-Díaz et al., 2001; Manchado et al., 2016; Padrós et al., 2011). There are numerous studies in flatfish about the anatomical and functional maturation of multiple organs and regulatory pathways behind the morphological changes (Alves et al., 2016; Guerrero-Peña et al., 2024; Louro et al., 2020). However, there is a notable lack of studies focusing on the maturation of the immune system.

The effects of THs as modulators of the immune system have been studied mainly in mammals, and key observations include: (1) their importance in thymus ontogeny and thymopoiesis (Geven and Klaren, 2017); (2) the increased susceptibility of hypothyroid mice to infections (Dorshkind and Horseman, 2000); (3) altered cytokine expression in patients with thyroid disorders (de Vries et al., 2015; Heuer et al., 1996; Mikoś et al., 2014); and (4) TR and thyroid stimulating hormone receptor (TSHR) expression by thymocytes and mature lymphocytes in the spleen (Luo et al., 1989; Pekonen and Weintraub, 1978; Villa-Verde et al., 1992; Wang et al., 2003). Conversely, hypothalamus-pituitary-thyroid (HPT) axis cells detect pro-inflammatory cytokines like tumour necrosis factor-alpha (TNFα) or interleukin IL-1b and IL-6 produced by activated macrophages (Zheng et al., 1991) and various steps of thyroid hormonogenesis can be impaired by these humoral factors (Geven and Klaren, 2017). Challenging the conventional HPT-axis model for TH synthesis in mammals, macrophages, monocytes, and neutrophils, also produce TSH and TH in response to alloantigens or viral exposure (Geven and Klaren, 2017; Quesada-García et al., 2014; Verburg-van Kemenade et al., 2017).

In fish although relatively few studies exist there is some evidence supporting a role for THs in immune system ontogeny such as the maturation of innate immune physical barriers (e.g., skin and mucous) during TH-driven metamorphosis (Alves et al., 2018; Campinho et al., 2012; Klaren et al., 2008; Kulkeaw and Sugiyama, 2012; Ponce et al., 2011). In killifish, hypothyroidism reduced the number of circulating leukocytes (Slicher, 1961), a phenomenon that also occurred and could be reversed by administration of TSH or thyroxine (T4) in guppies (Ball and Hawkins, 1976). Leukocytes like macrophages, lymphocytes, and granulocytes take up and convert T4 to Triiodothyronine (T3) via specific transporters in zebrafish (Arjona et al., 2011; Geven and Klaren, 2017). Additionally, vertebrate somatic cells, including immune cells, expressed one or more TR (Villa-Verde et al., 1992), a fact confirmed in rainbow trout (*Oncorhynchus mykiss*) (Quesada-García et al., 2014) and zebrafish (*Danio rerio*) (Heijlen et al., 2013).

The anatomical co-localization of thyroid follicles and the haematopoietic-lymphoid tissue of the head kidney has led to speculation about a link between these two tissues (Geven and Klaren, 2017). Moreover, the variable activity of heterotopic renal thyroid tissue compared to subpharyngeal thyroid tissue, makes the physiological relevance of THs in immune tissue uncertain (Bhattacharya et al., 1976; Chavin and Bouwman, 1965; Frisén and Frisén, 1967; Peter, 1970). In this context the aim of the present study was to determine if THs contribute to immune system development in teleost fish. The Senegalese sole was chosen as the model since it has a well characterized and easily identified metamorphosis driven by THs. Transcriptomics was used to identify immune genes activated in different developmental stages of larvae. The likely regulation by THs of immune genes with a modified expression during metamorphosis was determined by searching for TREs in their promoter regions and the TRs expressed during metamorphosis. Overall, insight was gained into the crosstalk between the thyroid axis and the cellular and humoral immune repertoire in the Senegalese sole and hypothesis raised for future investigation.

2. Methodology

All animal manipulation and procedures complied with the EC Directive 86/609/EEC for animal experimentation, and Spanish regulations for animal welfare. All procedures were approved by the Animal Ethics Committee of IFAPA (Andalusian Institute of Agricultural, Fisheries, Agrifood and Organic Production Research and Training) and observed international guidelines for best practice outlined in the 3R ethical principles for planning and execution of animal experiments.

2.1. Sole rearing and larvae sampling

To investigate the development of the immune system in sole larvae, eggs were obtained from naturally spawning Senegalese sole broodstock (IFAPA Centro El Toruño). Fertilized eggs were selected by buoyancy: the viable buoyant eggs were collected while the non-viable, non-buoyant eggs, were discarded. The eggs were incubated in 15 L cylindrical tanks (3000 embryos L^{-1}) in an open seawater circuit with gentle aeration. Newly hatched larvae at 1-day post-hatch (dph) were transferred to a 400 L tank (45–50 larvae L^{-1}). For detailed sole larval rearing protocols see Manchado et al. (2008). Briefly, at the onset of external feeding when mouth opening occurred (3 dph), larvae were fed with *Tisochrysis lutea* (T-iso strain) enriched rotifers (*Brachionus plicatilis*) until 9 dph. Thereafter and until the end of the experiment (20 dph) larvae were fed with enriched artemia (*Artemia metanauplii*). The mean water temperature and salinity were 21.1 ± 0.3 °C and 34.2 ± 0.2 ppt, respectively and a photoperiod of 16 h light:8 h dark was used.

Larvae were sampled at different developmental stages as previously described (Fernández-Díaz et al., 2001): pre-metamorphic (D7, 7 dph), onset of metamorphosis (S0, 12dph), metamorphosis (S1, 14dph), early climax (S2, 15 dph), climax (S3, 16 dph) and post-metamorphic juveniles (S4, 20 dph). For sampling, larvae were euthanized in MS-222, washed using diethylpyrocarbonate (DEPC)-treated water, snap frozen in liquid nitrogen, and stored at −80 °C until analysis. The sampling points were selected based on previous studies carried out on sole at IFAPA, relating days post-hatch, external morphology and the typical change in TH concentrations in Senegalese sole larvae, namely low concentrations of THs until 9 dph, a peak of THs at around 15 dph (S2) and low THs in 17 dph (S3) post-metamorphic animals (Manchado et al., 2008) (Fig. 1).

2.2. RNA extraction and cDNA library preparation

RNA was extracted from pooled larvae collected at six developmental stages: D7, S0, S1, S2, S3, and S4. For each stage, larvae were sampled from three independent replicate tanks (n = 3). From each tank, one pooled sample of larvae was collected (n = 1), resulting in a total of 18 samples. The number of larvae in each pool was adjusted to yield the RNA amount required for sequencing. Briefly, larval pools were homogenised using a Fast-prep FG120 instrument (Bio101) and lysing Matrix D (Q- Bio-Gene) for 40 s at a speed setting of 6. Total RNA (>200 nucleotides) was isolated using a RNeasy Mini Kit (Qiagen) following the manufacturer's indications and treated twice with DNase (RNase-Free DNase kit, Qiagen) for 30 min to digest single and double-stranded DNA. RNA was quantified with a NanoDrop 8000 spectrophotometer (Thermo Scientific) and its quality was assessed by 2 % agarose gel electrophoresis. Illumina cDNA libraries were constructed and sequenced following the manufacturer's protocol as previously described (Benzekri et al., 2014). The cDNA library dataset was submitted to the Sequence Read Archive (SRA) and the submission codes and sample identification are indicated in Supplementary Table S1.

2.3. Gene expression analyses.

To assess differential gene expression, libraries were pre-processed using SeqTrimBB v2.1.8 and cleaned reads were mapped onto the

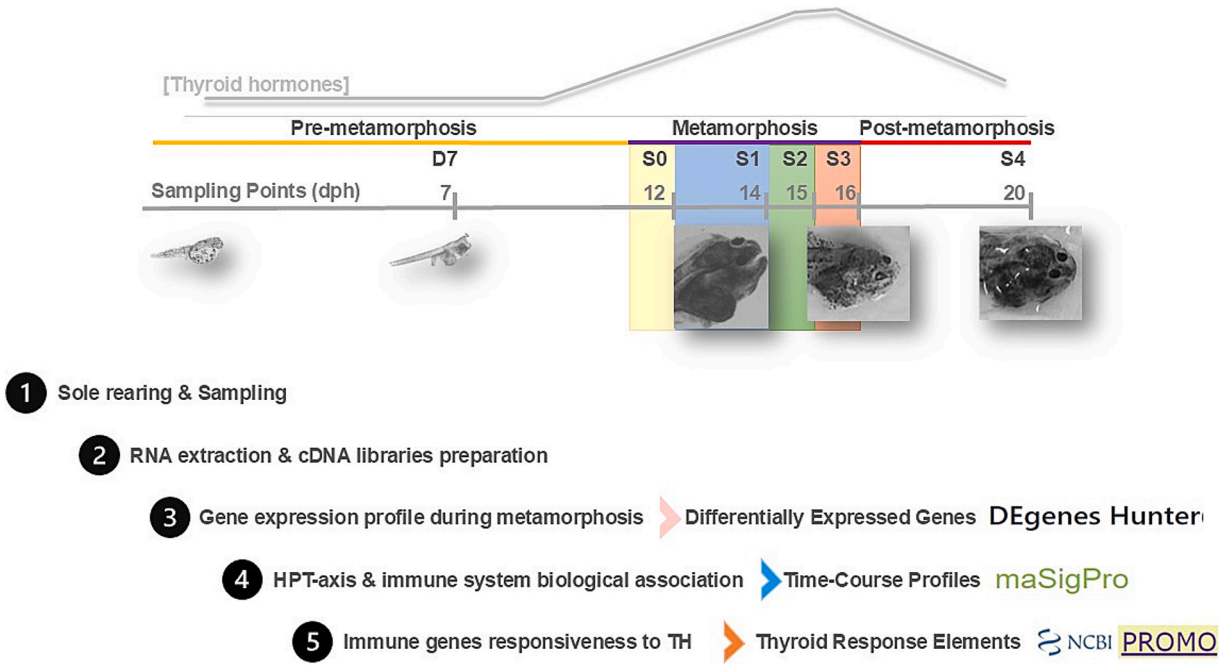


Fig. 1. Schematic diagram of the experiment and approach taken for transcriptome analysis. Solea senegalensis larvae were sampled (n = 3 larval pools) at different metamorphic stages: before metamorphosis at 7 dph (D7), at metamorphosis onset (S0), during metamorphosis with different degrees of eye migration (S1, S2, and S3 according to eye migration progress), and at the end of metamorphosis when eye migration is completed (S4). A total of 18 samples were collected (n = 3 per developmental stage). The pattern of THs in whole animals was taken from Manchado et al. (2008).

Solea senegalensis transcriptome (Guerrero-Cózar et al., 2021) using BBmap v38.92 (Bushnell, 2014). The number of reads appropriately mapped was extracted using the sam2counts program from the SAMtools suite (Li et al., 2009) (https://github.com/vsbuffalo/sam2counts). For differential analysis, the number of reads was normalized, and statistical analysis was performed using edgeR and DEseq2 methods implemented in the DEGenesHunter program (https://github.com/Isabelggayte/DEgenesHunter) (Jabato et al., 2021).

Differentially expressed transcripts (DETs) between the D7 stage and developmental stages S0, S1, S2, S3, and S4, as well as between S0 and stages S2, S3, and S4, with an FC > 1.5 (FC: relative change in expression between groups) and a false discovery rate (FDR) < 0.05 between groups were considered significant. For a given comparison, the results are shown as mean logFC. Positive values indicate gene upregulation while negative values indicate gene downregulation. Time-course gene expression pattern clustering analysis was performed using MasigPro software (Conesa et al., 2006). A heatmap was generated in R version 3.2.2 using the heatmap.2 function from the gplots package (https://cran.r-project.org/web/packages/gplots/index.html). Functional enrichment analysis was carried using the ClueGO v2.5.8 Cytoscape v3.8.2 plug-in (Bindea et al., 2009).

2.4. Gene promoter analysis to Identify TR binding sites

To identify thyroid-response elements (TREs), the promoter sequence of differentially expressed immune-related genes were obtained from the Senegalese sole genome available at NCBI (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_019176455.1/). Based on assumptions about the approximate location of promoter sequences in fish genes, a 1000 bp region upstream of the transcription start site of each target immune-related gene was analysed (Yen, 2001). PROMO software (Farré et al., 2003; Messeguer et al., 2002) was used to conduct *in-silico* identification of putative TREs and retinoid X receptor (RXR) DNA binding sites in the promoter region of genes, employing a similarity threshold of 85 %. PROMO utilizes the TRANSFAC database v8.3 to generate specific binding site weight matrices for predicting transcription factor binding sites. Only eukaryote factors and sites were included in the analysis. The T3R-alpha [T00841], T3R-alpha [T01351], T3R-beta [T00852], T3R-beta1 [T00853], RXR-alpha [T01345], RXR-beta [T01349] and RXR-beta [T01332] transcription factors were included in the search. Subsequently, the DNA sequences obtained for each transcription factor was aligned using MultAlin software (Corpet, 1988) to identify consensus sequences.

3. Results

We conducted comprehensive transcriptome analysis using RNA-seq to explore gene expression patterns across different developmental stages of Senegalese sole larvae. The 18 sequencing libraries (n = 3 for each of the six developmental stages studied) generated a mean read number of 23.7 million, with a mean overall alignment rate to the reference *S. senegalensis* male genome of 84.2 %. The cleaned and mapped data for each sample, are provided in Supplementary Tables S2 and S3, respectively.

3.1. The gene expression fingerprint is distinct before and after metamorphosis

The heatmap generated using DETs in premetamorphic, metamorphic and postmetamorphic *S. senegalensis* larvae is depicted in Fig. 2. Sample clustering clearly separated different metamorphic stages except those just at the onset of metamorphosis (S0 and S1). The premetamorphic stage D7 (7 dph) and the post-metamorphic S4 stage (20 dph) exhibited unique gene expression patterns, which set them apart in the clustering analysis. The metamorphic stages, S0 – S3 formed subclusters under a single branchpoint indicating less divergence in the gene expression pattern between these samples.

The transition from a young pelagic larva (D7) to a larva just at the onset of metamorphosis (S0) corresponded to a massive-gene-oriented developmental process (Fig. 3A). Comparing pre-metamorphic 7 dph larvae (D7) with 12 dph larvae (S0), 5993 DEGs were identified of which 2470 transcripts were down-regulated, and 3523 up-regulated (Fig. 3A).

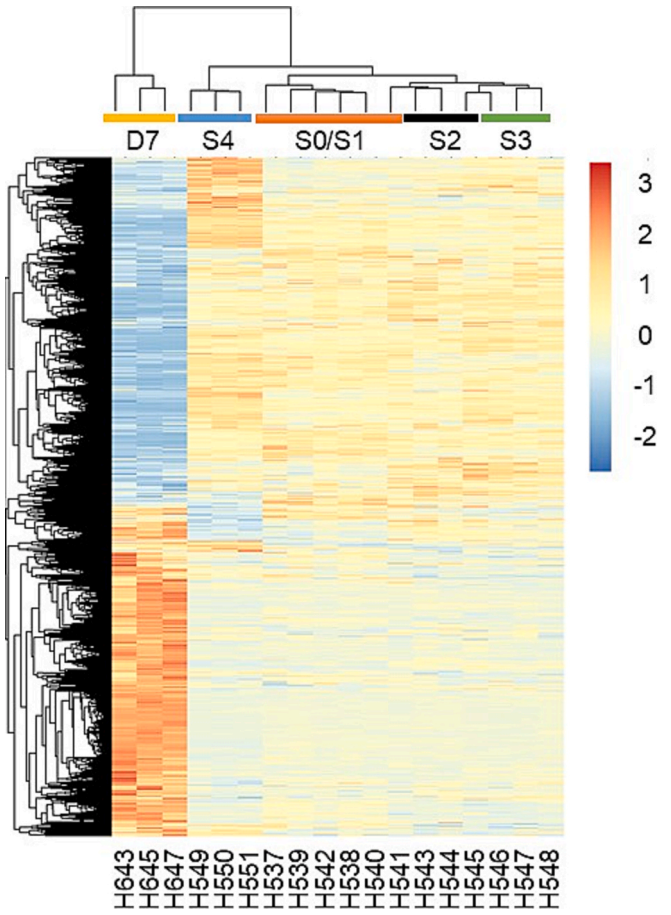


Fig. 2. Heatmap of DETs during larval development. D7 = pre-metamorphic larvae (7 dph); S0 = metamorphosis onset (12 dph); S1 = metamorphosis (14 dph); S2 = early climax (15 dph); S3 = climax (16 dph); S4 = post-metamorphic juveniles (20 dph). Samples from each of the six developmental stages were collected from triplicate experimental tanks (n = 3 samples/stage). Transcripts with a fold change > 1.5 and FDR < 0.05 were considered to have a significantly modified expression. The orange to red colour gradient indicates high relative abundance (up-regulation). The blue colour gradient indicates low relative abundance (down-regulation) and the yellow colour indicates equal abundance. The clustering differentiated the stages D7, S2, S3 and S4 but not S0 and S1. Heatmap clustering was performed using the Ward.D2 method and generated with heatmap.2 on normalized transcript read counts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Comparisons of the 7 dph larvae, D7, with the metamorphic larvae, S1, S2 and S3 yielded 7221, 7161, and 7451 DETs for each stage, respectively. Comparison of D7 larvae (7 dph) with the post-metamorphic juveniles (S4, 20 dph) yielded 8145 DEGs. Functionally (Supplementary Tables S4.1–S8.1), several regulatory pathways enriched in genes linked to maturation of the brain, neurogenesis, development of the central and peripheral nervous systems, heart, regulation of immune system, circulatory system, eye, pigmentation, and pancreatic endocrine cells were identified. At a molecular level, this post-embryonic developmental maturation was accompanied by the expression of genes involved in DNA replication, repair, and transcription, RNA splicing, protein synthesis, degradation, and post-transcriptional modifications. Given the intense developmental activity, there was upregulation of the genetic toolbox responsible for regulating cell populations, and maintaining cellular homeostasis, and apoptosis.

The onset of metamorphosis and the two subsequent sampling days 12 dph (S0) and 14 dph (S1) exhibited a similar gene expression fingerprint. Overall, during this developmental period, genes related to the developing digestive tract, adipose tissue, bone, cartilaginous tissue, and body axis asymmetry were upregulated (Supplementary Tables S4.2 and S5.2). At 15 dph, S2, modification in expression of genes related to the development of the fins, eyes, and cardiac muscle were evident, while genes related to the bones and craniofacial formation continued to be modified at this stage (Supplementary Table S6.2). Pathways for the development of endothelial cells, melanocyte migration, and neuron projection showed increased gene expression during the metamorphic climax (S3) (Supplementary Table S7.2). Throughout metamorphosis, genes associated with distinct metabolic and enzymatic activity pathways were identified and contributed to distinguish the gene fingerprints of the metamorphic S0/S1, S2, and S3 stages from the D7 pre-metamorphic larvae.

In post-metamorphic larvae S4 (Supplementary Table S8.2) notable features included the development of otoliths, epidermis, fins, and reproductive processes, along with the morphogenesis of dendrites and bones. At the molecular level, processes such as DNA replication, repair, and transcription remained crucial. Additionally, some epigenetic mechanisms, such as post-transcriptional regulation of gene expression and histone modification, were also observed. The expression of genes involved in the positive regulation of leukocyte activation, NF-kappaB transcription factor activity, and chemokine activity further distinguished the post-metamorphic stage (S4) from other developmental stages (Supplementary Table S9).

3.2. TH-related genes were differentially expressed from the onset of metamorphosis

Confirmation of the robustness of the transcriptome dataset and insights into HPT axis activation during metamorphosis was gained from the analysis of the expression of genes related to this axis. The pre-metamorphic developmental stage of sole larvae was characterized by a significant expression of two isoforms of *pax8* and *trhr* that had reduced mRNA levels during metamorphosis. In contrast, a significant increase in the expression of core genes involved in TH synthesis – such as *tgb* (key for TH production in thyroid follicles), TH transformation – such as *mct8* (mediator of cellular TH uptake) and *dio1* (catalyses both activation and inactivation of THs) – as well as the nuclear receptors *tra* and *trb*, was observed across sole metamorphosis (Supplementary Table S10). Notably, some HPT axis-related genes were constitutively expressed from 7 dph (D7) until the completion of metamorphosis (S4). This included *trh*, *tsh* and *tshr*, which are key factors regulating TH production. The same constitutive expression pattern was observed for thyroid peroxidase (*tpo*) and the sodium/iodide symporter (*nis*) genes, pivotal for TH synthesis, as well as the cellular TH transporter, *slc7a5* (Supplementary Table S11).

Two orthologous genes that code for the Dio1 enzyme were identified (Supplementary Fig. 2). One of these genes, SSENm1B013238T1, was constitutively expressed from pre-metamorphosis onwards and exhibited a high relative expression level. The gene SSENm1B027161T1 was upregulated at the onset of metamorphosis (S0) and was the only deiodinase gene showing differential expression during sole metamorphosis (S1–S3) and in post-metamorphic larvae (S4; Supplementary Tables S10 and S11). Additionally, two orthologous genes encoding Dio2 (SSENm1B049115T1 and SSENm1B009745T1) and two paralogous genes encoding Dio3 (SSENm1B000391T1 and SSENm1B039461T1) were low abundance, and their expression was not significantly different during sole metamorphosis (S0–S4).

Two *tra* genes (*traA* and *traB*) and two *trb* genes (*trb* and *trb2*) were expressed in sole metamorphic larvae and *traA* (SSENm1B038213T1) and *trb2* (SSENm1B027317T1) genes were upregulated from the onset of metamorphosis until the climax (*traA*) or in post-metamorphic juveniles (*trb2*) (Supplementary Table S10). The *traB* gene was highly and continuously expressed from D7 (7 dph), while the *trb* gene exhibited low and not significantly different expression levels across

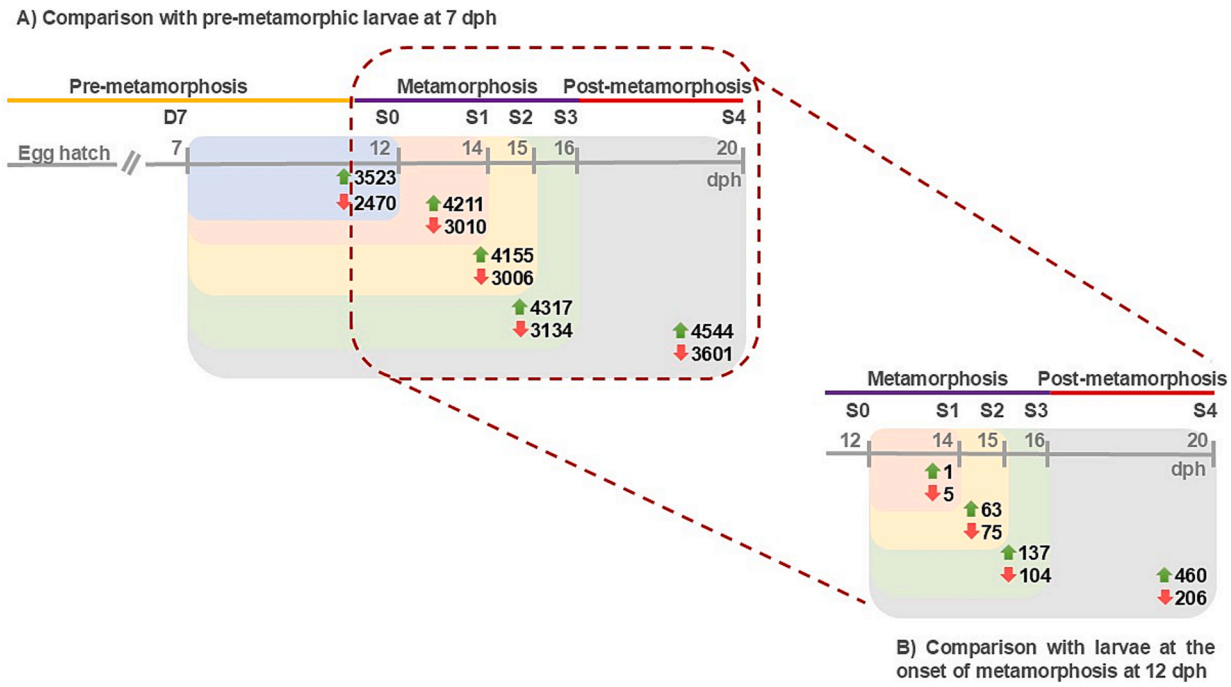


Fig. 3. Differentially expressed Transcripts (DETs) were identified by (A) comparison of the transcriptome of the D7 larvae (7 dph) with the transcriptome of each of the metamorphic stages (S0, S1, S2, S3) and with the post-metamorphic juvenile (S4), or (B) comparison of the transcriptome of larvae at the onset of metamorphosis (12 dph, S0) with each of the metamorphic stages (S1, S2, S3) and with the post-metamorphic juvenile (S4). Transcripts with a fold change > 1.5 and FDR < 0.05 were considered significant. Down-regulated genes are indicated by red arrows and upregulated genes are indicated by green arrows. The number of DETs resulting from each comparison is indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metamorphosis (Supplementary Table S11). Two isoforms of the nuclear receptor corepressor 1 and nuclear receptor coactivator 1, which interact with TR and modulate the transcriptional effects of THs, had a balanced and constant expression throughout sole metamorphosis (Supplementary Table S11).

3.3. Total transcriptional activity is stable across metamorphosis

Transcriptomic responses between larvae at the onset of metamorphosis (S0) and those in metamorphosis were less pronounced compared to the differences observed with respect to young larvae and larvae at various stages of metamorphosis. Such differences increased as metamorphosis progressed (Fig. 3B). The number of DETs between S0 and S1 and S2 was 6 and 138 genes, respectively. Comparison of S0 with S3 (metamorphic climax) yielded 241 DETs and S0 compared to S4 (post metamorphic juveniles) yielded 666 DETs. Functionally, the pathways enriched at metamorphic climax were mainly related to metabolism (retinoid metabolic process), enzymatic activity (threonine-type peptidase and cytochrome-c oxidase), homeostasis (of metal ions and sterol), and development of the heart and circulatory system (Supplementary Table S12).

3.4. Immune system ontogeny during metamorphosis

To elucidate immune system ontogeny during sole metamorphosis, the functional relevance of DETs was assessed. Genes involved in the regulation of the immune system, definitive (or adult) haematopoiesis (differentiation into different blood cell lineages), kidney development, and lymphocyte activation were down-regulated in the metamorphic stages (S1 to S3) compared to the pre-metamorphic stage, D7 (Supplementary Table S9 and Fig. 4). Genes involved in thymus development, wound healing, granulocyte migration, and cytokine response were downregulated in post-metamorphic sole juveniles (S4).

The onset of metamorphosis was marked by the upregulation of genes involved in various immune-related processes, including the regulation of granulocyte chemotaxis and immune responses mediated by cell surface receptors. Additionally, genes related to the migration of hematopoietic stem cells, scavenger receptor activity, and antigen processing and presentation via MHC class I molecules showed increased expression. GO enrichment analysis indicated that the liver underwent modifications during the transition from larva to juvenile. Upregulation of genes associated with lymphocyte differentiation and natural killer cell activity also occurred during metamorphosis.

Regarding cell differentiation, two mechanisms suggest a suppression of this process. On one hand, there was downregulation of genes involved in haematopoiesis, while, on the other hand, genes involved in the negative regulation of hematopoietic progenitor cell differentiation were upregulated. Some genes associated with enriched immune processes, such as antigen processing and presentation via MHC class I and the negative regulation of hematopoietic progenitors, were upregulated throughout sole metamorphosis. Other pathways emerged at specific metamorphic stages, such as natural killer cell activation (S1), cytokine receptor activity, and the defence response (both during S2), and persisted until post-metamorphosis. Tumour necrosis factor-mediated signalling was specific to the S1 and S2 stages. Overall, metamorphosis was associated with the development of a comprehensive immune system repertoire in sole.

3.4.1. Hematopoietic multipotency, myeloid and lymphoid biosignature genes during metamorphosis

The analysis of hematopoietic process markers revealed significant changes during metamorphosis (Fig. 5 and Supplementary Table S13). Expression of hematopoietic stem cell markers, macrophage and dendritic cells, natural killer cells and T lymphocytes and key genes for generation of T and B cell receptors were up-regulated.

3.4.2. Soluble innate immunity mediator genes during metamorphosis

A total of 24 deduced soluble innate immune mediators corresponding to 45 DETs were identified (Fig. 6 and Supplementary Table S14). Thirteen out of the fourteen DETs for acute-phase proteins

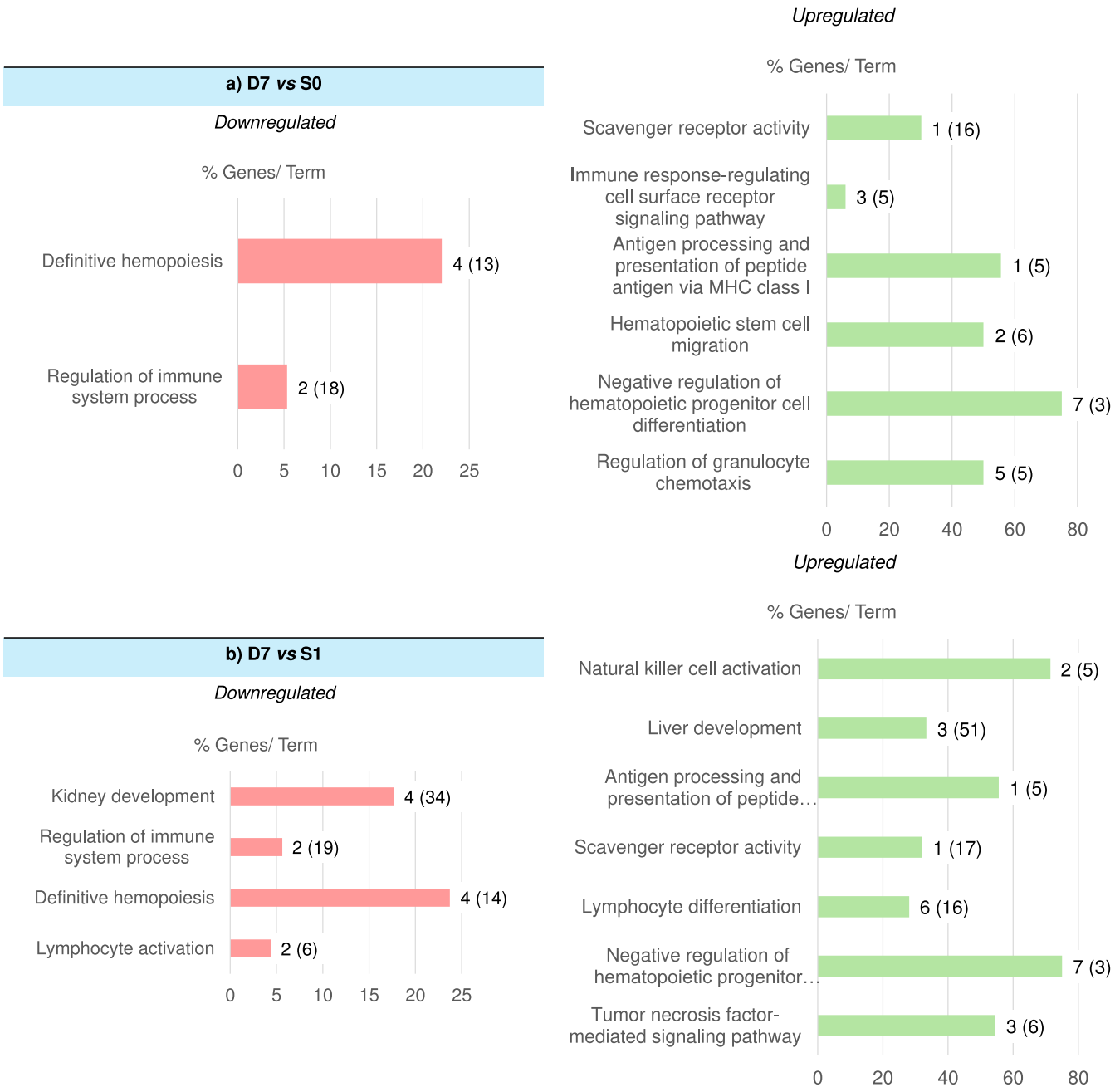


Fig. 4. Bar plots depicting the most significant up- and down-regulated Gene Ontology (GO) terms associated with the immune system and represented by the differentially expressed genes, along with the percentage of DEGs present in each functional pathway (term), when comparing D7 (7 dph) larvae with metamorphic larvae and juveniles. Numbers at the end of the bars are the enriched pathways and the genes/term are indicated in brackets. a) Comparison of D7 (7 dph) and S0, onset of metamorphosis; b) Comparison of D7 and S1, early metamorphosis; c) Comparison of D7 with S2 mid-metamorphosis; d) Comparison of D7 with S3 metamorphic climax, and e) Comparison of D7 with S4 post-metamorphosis (juveniles).

were up-regulated during metamorphosis (S0 onwards), while others, such as lysozyme (*lys*) g-like genes (SSENm1B026463T1) and ferritin heavy chain (*fth*) genes, were upregulated at the metamorphic climax (S3) and post-metamorphosis (S4).

3.5. Concordant expression patterns of immune and HPT axis-related genes

We used maSigPro to identify statistically significant differential expression profiles from the time-course metamorphosis data, which yielded 2673 DETs classified into nine clusters (Supplementary Fig. 1, Supplementary Table S15).

Co-expressed genes are often co-regulated, meaning their transcription is activated or suppressed by the same transcription factors or signalling pathways. Clustered genes may also participate in related biological processes, working together in response to specific cues or as part of a coordinated program that ensures proper development (Godichon-Baggioni et al., 2019). Clusters 2, 3, 4, 5 and 7 had a concordant expression pattern between HPT-axis genes and immune-related genes (Fig. 7). TH genomic action-related genes, including nuclear receptors *traA* and *trb*, deiodinase *dio1*, and the co-repressor *n-cor2*, were transcriptionally synchronized with genes involved in lymphoid

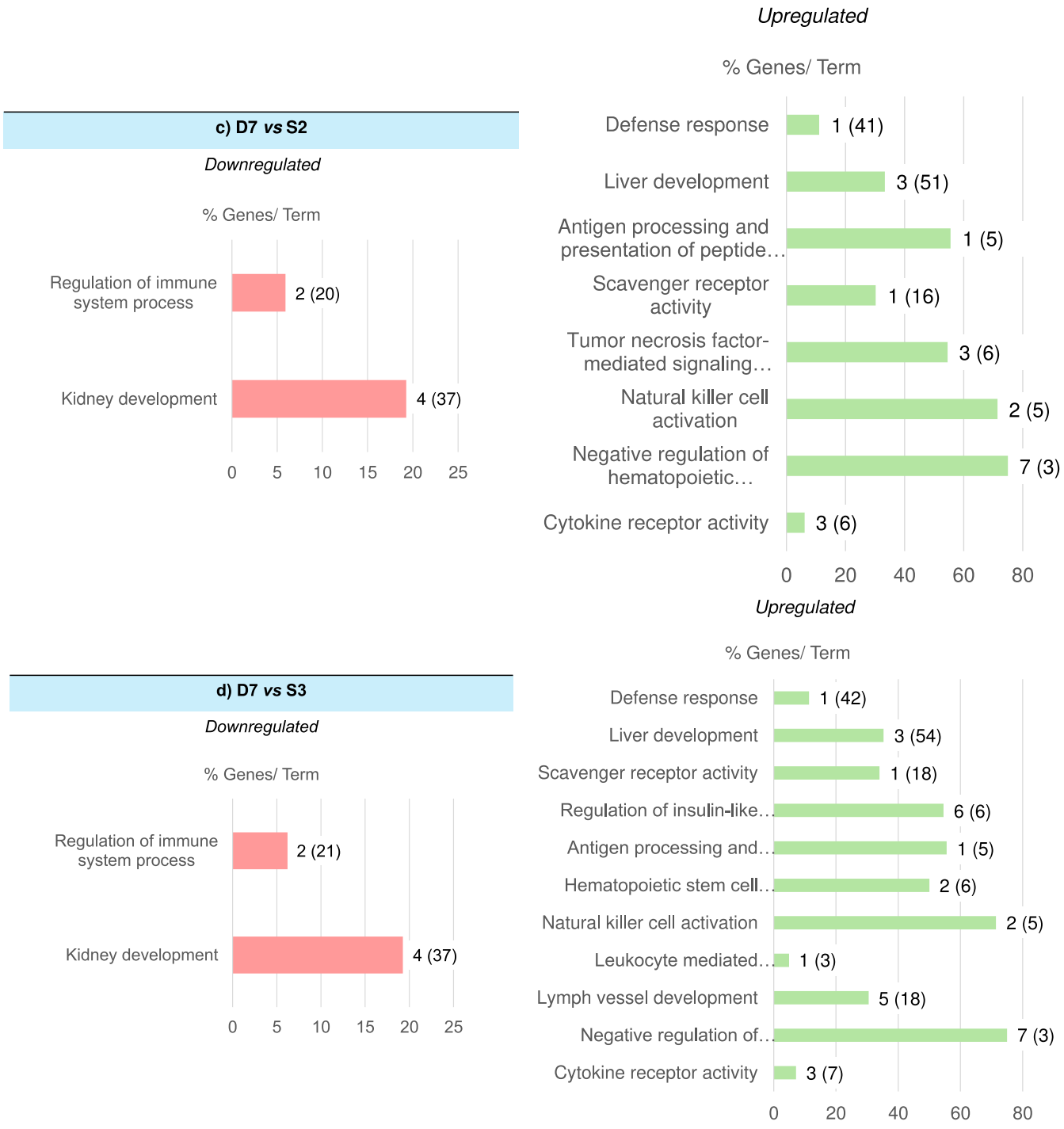


Fig. 4. (*continued*).

organ maturation (T-cell differentiation in the thymus), haematopoiesis (somatic recombination and markers for macrophages, dendritic cells, mast cells, natural killer cells, and B lymphocytes), and humoral immunity (cytokines, chemokines, acute phase proteins, and complement system components).

3.6. Thyroid responsive immune-related genes inferred from the presence of thyroid-response elements

Analysis of promoters for 133 DETs related to the immune system (including those with coincident expression patterns with HPT-axis-related genes) indicated that 84 of the 133 analysed genes contained putative TREs within a 1000 bp region upstream of the open reading frame (ORF) (Supplementary Table S16). Specifically, one distinct DNA binding site for TRα (*nt*g*n*G*n*t*C*a*c*a*n*) was identified and a consensus nucleotide sequence (*nnn*tGgtCa*nnn*) was found that likely can bind either TRα or TRβ interchangeably (Fig. 8). It was noted that core immune-related transcripts, including *cxcl12* (chemotaxis), *csf1r* (macrophage development), *pglyrp6* (peptidoglycan recognition), *rag1* (V(D)J recombination), and *c3a.1*, *c3a.4*, and *c2* (complement system), lacked TR binding sites in their proximal promoter regions. The putative TR candidate binding sites characterized in the immune-related transcripts that were DE across metamorphosis are listed in Supplementary Table S16.

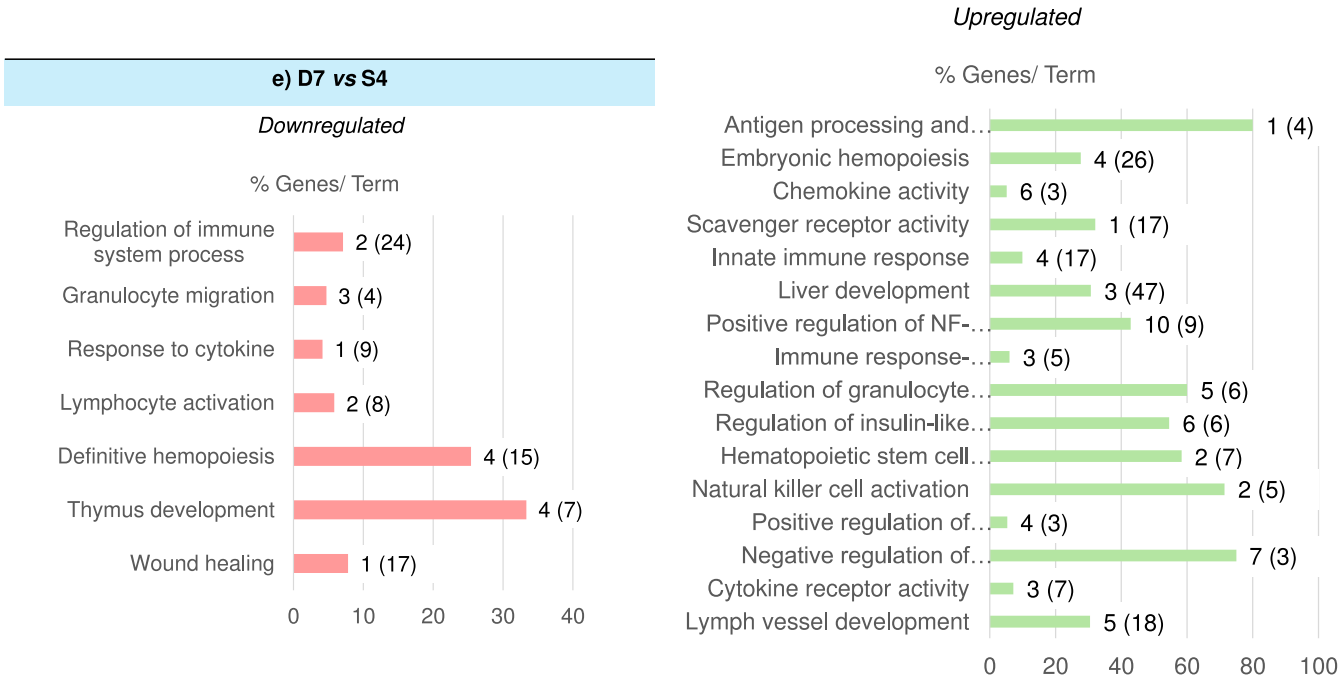


Fig. 4. (*continued*).

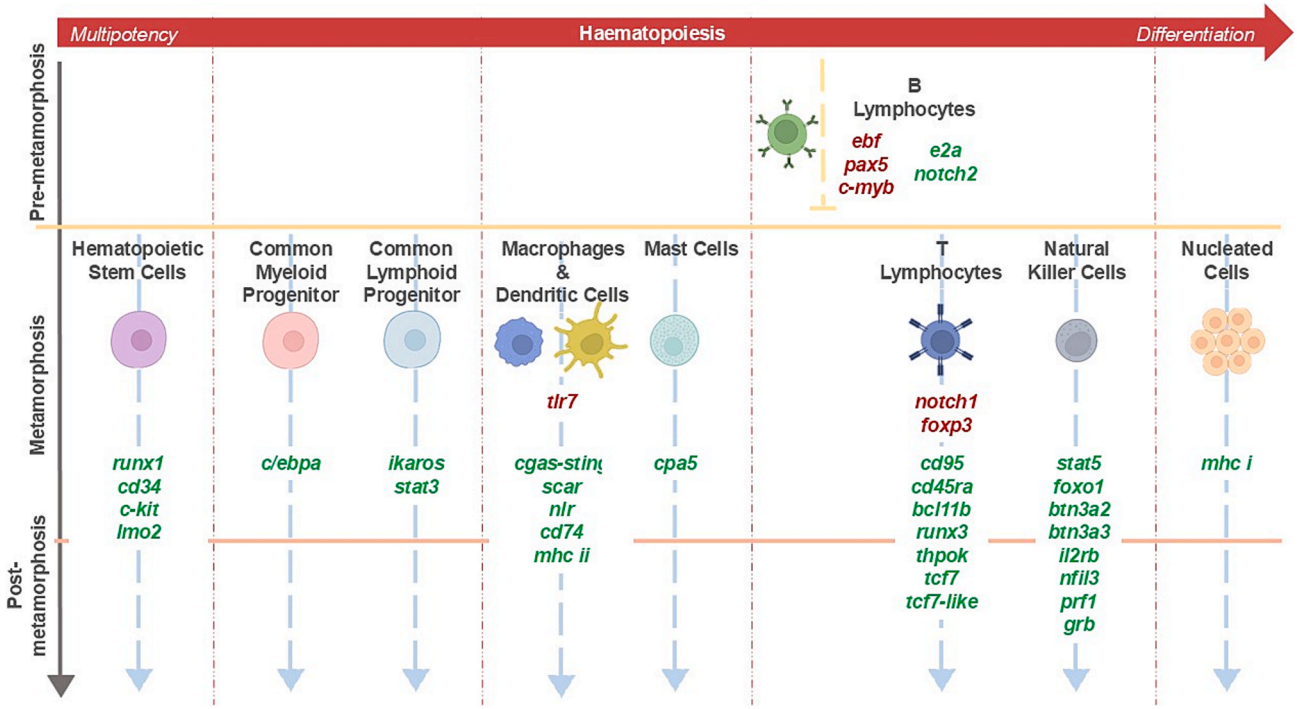


Fig. 5. Schematic representation of hematopoietic gene biomarkers identified as differentially expressed (DE) during metamorphosis of Senegalese sole larvae. Three developmental stages (pre-metamorphosis, metamorphosis, and post-metamorphic juveniles) are shown, along with DETs associated with specific immune cell specialization (multipotency *vs* differentiation) and lineage (myeloid *vs* lymphoid). Up-regulated transcripts are highlighted in green, while down-regulated transcripts are in red. The gene expression cluster to which each transcript belongs are indicated in Supplementary Table S13. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To enhance binding stability at TREs, TRs can form heterodimers with RXRs. The promoter regions analysed contained RXR β-specific DNA binding sites, which were less abundant than TREs (Supplementary Table S16). Alignment of multiple sequences unveiled two putative consensus sequence for RXRβ (gtcAgTgac and *nnn*agGtCat*nnn*) (Fig. 8). Notably, only RXRβ binding sequences were present in the analysed promoter sequences, except for *loxl2a* (scavenger activity), which contained one RXRα binding site.

Regarding the DE immune transcripts with TREs in their promoter regions, some down-regulated genes, such as *cacna1fb* and *kcnj11l* (involved in regulating immune-related processes), and *smarcd1* (definitive haematopoiesis), as well as the up-regulated *efna2a* (involved

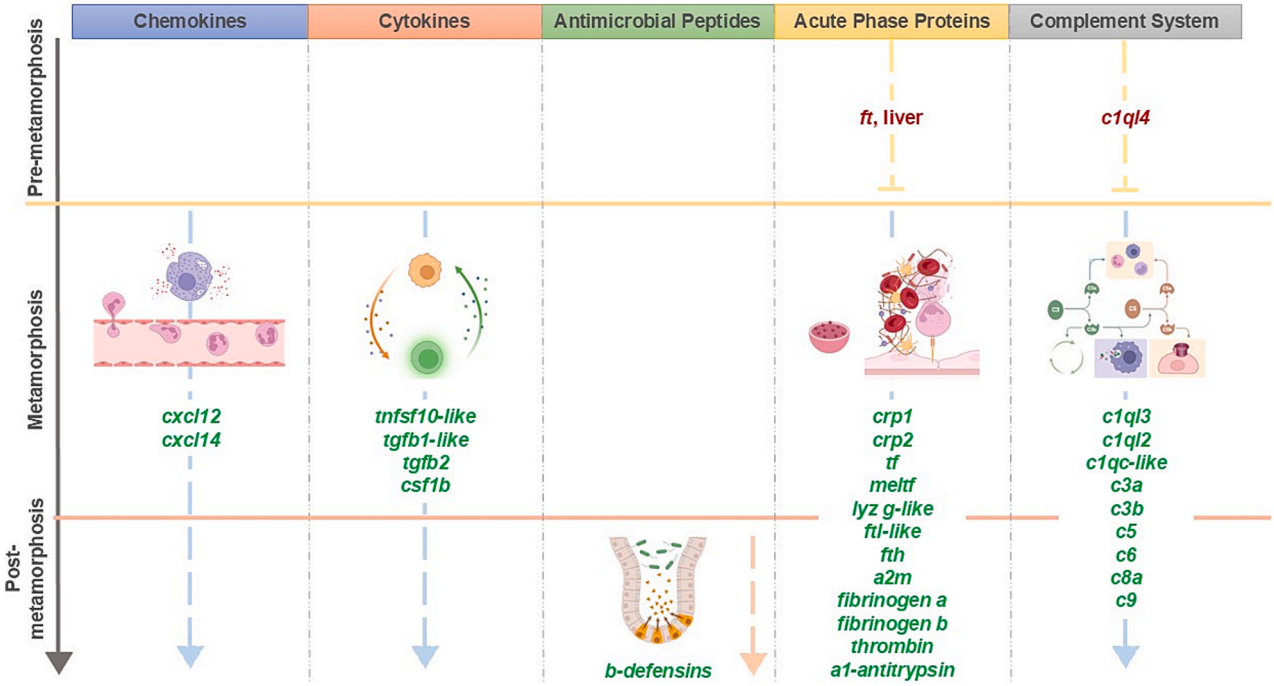


Fig. 6. Schematic representation of differentially expressed (DE) innate immune humoral factors during Senegalese sole larvae metamorphosis. Humoral factors are listed in their general categories: chemokines, cytokines, antimicrobial peptides, acute-phase proteins, and complement system elements. The gene expression clusters to which each transcript belongs are indicated in Supplementary Table S14. Abbreviations: cxcl, Chemokine (C-X-C motif) Ligand; *tnfsf*, Tumour Necrosis Factor Superfamily; *tgf-b*, Transforming Growth Factor β; *csf*, Colony-Stimulating Factors; *crp*, C-reactive protein; *meltf*, Melanotransferrin; *lyz*, Lysozyme; *a2m*, Alpha-2-Macroglobulin.

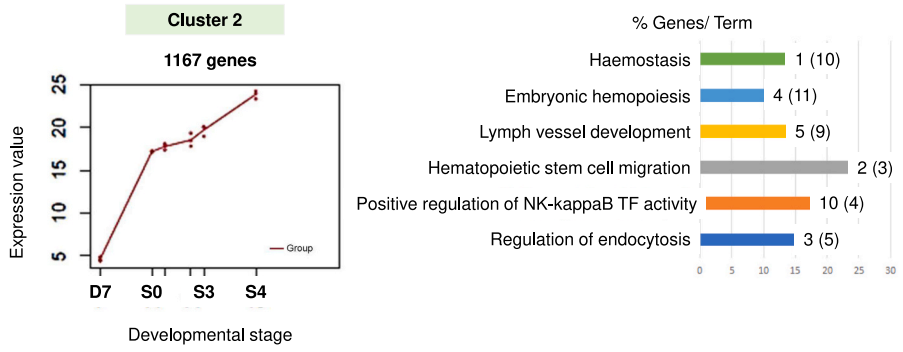
in natural killer cell activation), exhibited a high density and diversity of TR binding sites. Other DE immune transcripts, such as the down-regulated *cish*, *crhb*, and *gata3* (immune regulation), and *pbx1a* (hemopoiesis), as well as the upregulated *rac2* (granulocyte chemotaxis), *irf7* (haematopoiesis), *gpr1* (immune receptor signalling), and *enpp1* (scavenger activity), each contained only one TR binding site, along with few or no RXRβ binding sites. Notably, gene promoters with a low abundance of TREs always contained the interchangeable TR α and β binding site sequence. In each DE immune-related pathway, there was at least one transcript without TR binding sites in their promoter. None of the transcripts that constitute the positive regulation of the complement immune response pathway (*c3a.1*, *c3a.4*, and *c2*) had TREs or RXR binding sites in their promoter regions.

4. Discussion

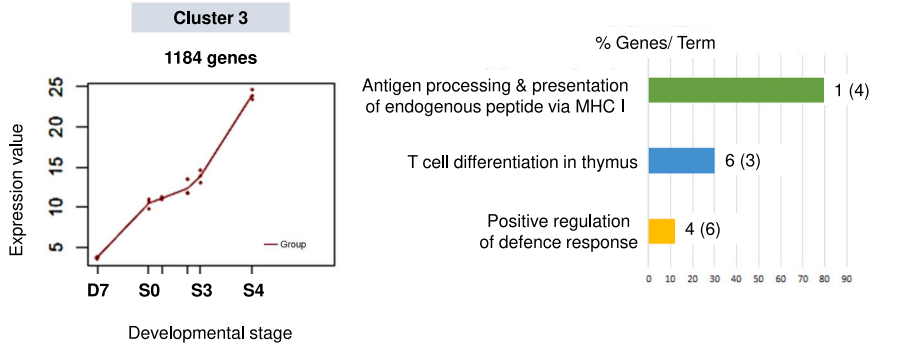
This study used transcriptomics to investigate how immune-related genes are regulated during metamorphosis, a TH-driven process, in Senegalese sole. A close regulatory relationship between the thyroid and immune system was established by identifying DE transcripts across metamorphosis, clustering thyroid and immune-related genes and identifying TREs in the promoter sequence of clustered candidate genes. Gene expression patterns indicated that the innate immune system matures during sole metamorphosis, although gene signatures for both innate and adaptive immune development were identified in pre-metamorphic larvae.

The results of the analysis of the TH axis were similar to previous studies of flatfish and confirmed the quality of the samples and the robustness of the sequencing and analyses. *Solea senegalensis*, like other species with pelagic eggs, has late thyroid differentiation, that starts after complete yolk resorption (Deane and Woo, 2003; Delgado et al., 2006; Padrós and Crespo, 1996; Specker, 1988; Tanaka et al., 1995). Critical transcription factors for thyroid development *pax8* and *foxe1* (Civitareale et al., 1989; Plachov et al., 1990; Raterman et al., 2023; Wendl et al., 2002; Zannini et al., 1997) were significantly up-regulated in our study before metamorphosis, which is consistent with previous histological findings that thyroid follicles begin to appear in sole, around 4 dph (Delgado et al., 2006; Wendl et al., 2002), and then increase in number thereafter (Delgado et al., 2006; Groman, 1982). The classical mammalian model of HPT-axis regulation, in which TRH stimulates TSH leading to TH synthesis, is not consensual in fish. Teleosts lack a median eminence, which connects the hypothalamus to the pituitary via blood vessels, and instead hypothalamic neurons release neurohormones directly onto pituitary cells that secrete TSH (Geven and Klaren, 2017). The role of TRH remains uncertain (Kagabu et al., 1998; Larsen et al., 1998; Melamed et al., 1995), with evidence suggesting that CRH might regulate the thyroid axis in some teleost (Bernier et al., 2009; Campinho et al., 2015). In Senegalese sole larvae in the present study, constitutive expression of the *trh* gene was observed from 7 dph (D7), while *crh* was downregulated during metamorphosis. The expression pattern of *tgb* in Senegalese sole resembled the pattern of changing T4 levels (Manchado et al., 2008), and this observation suggests there is a lack of negative feedback on *trh* transcription by *tgb*. This could imply that TRH plays a less significant role in the HPT axis, as has previously been proposed by Iziga et al. (2010). Instead, inhibition appears to target *crh* gene expression, which aligns with what has been described in tetrapods (avians and amphibians), suggesting that CRH may be the ancestral apex of the thyroid axis, and that TRH assumed this role more recently in mammals (De Groef et al., 2003; Galas et al., 2009; Okada et al., 2009). In fish, TRH is proposed to regulate the synthesis and release of growth hormone, adrenocorticotropic hormone, and α-melanocyte-stimulating hormone, each contributing to growth, tissue remodelling, and pigmentation during metamorphosis (Barry and Grau, 1986; Kagabu et al., 1998; Lamers et al., 1991; Rotllant et al., 2000; Tran et al., 1989; Trudeau et al., 1992; Van Der Salm et al., 2004).

Genes involved in TH synthesis, which is obligatory for flatfish metamorphosis, such as *nis* and *tpo*, were expressed in pre-metamorphic larvae. The *tgb* gene that produces thyroglobulin, the substrate for TH



Thyroid Function	
TH Deiodination & Action	*iyd (1)*
Immune Response	
Hematopoietic Stem Cell Markers	*lmo2 (1)*
Myeloid Lineage Markers	*c/ebpa (1)*
Lymphoid Lineage Markers	*stat3 (1)*
T Lymphocytes	*thpok (1)*
Chemokines	*cxcl12 (3)*
Acute Phase Proteins	*crp1 (2); tf (1); meltf (2); lys g-like (2); fibrinogen-like (1); fibrinogen α (1); fibrinogen β (1); Thrombin (1); α1 antitrypsin (1)*
Complement System	*c3b (2); c5 (1); c8a (2)*



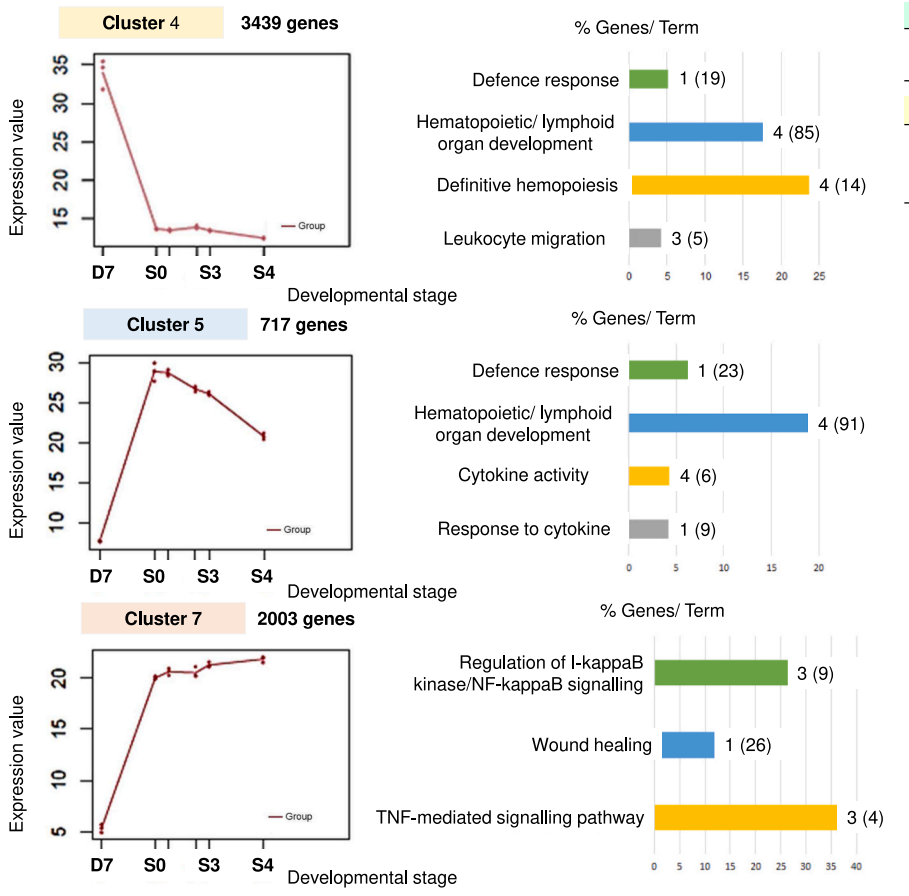
Thyroid Function	
TH Action	*trβ2 (1)*
Immune Response	
Hematopoietic Stem Cell Markers	*runx1 (1)*
Macrophage & Dendritic Cells	*cgas/ sting (1); nlr (19); cd74 (1); mhc ii (3);*
Lymphoid Lineage Markers	*ikaros (1)*
T Lymphocytes	*cd45ra (1); runx3 (1)*
Somatic Recombination	*rag1 (1)*
Natural Killer Cells	*btn3a3 (1); il2rb (1); nfil3 (1); prf1 (1); grb (1)*
Nucleated Cells	*mhc I (3)*
Chemokines	*cxcl14 (1)*
Cytokines	*tnfsf10-like (1)*
Antimicrobial Peptides	*β-defensins (1)*
Acute Phase Proteins	*fth (1)*
Complement System	*c1qc-like (1); c3a (8); c3b (1)*

Fig. 7. Gene expression clustering (rsq = 0.7) of DETs across metamorphosis in *S. senegalensis*. Genes in clusters 2, 3, 4, 5, and 7 exhibited co-expression of genes associated with the immune system and the hypothalamus-pituitary-thyroid (HPT) axis. Line graphs illustrate the gene expression pattern across metamorphosis. D7 = pre-metamorphic larvae (7 dph); S0 = metamorphosis onset (12 dph); S3 = metamorphic climax (16 dph); S4 = post-metamorphic juveniles (20 dph). The number of genes in each cluster is indicated in brackets. Histograms identify the most significant immune-related category per gene ontology (GO) group, and the percentage of associated genes. The number of pathways is indicated, and the number of genes is in brackets. Tables present annotation of gene transcripts and the number of transcripts for clustered HPT-axis and immune system-related genes.

production, was up-regulated from the onset of metamorphosis (cluster 5) as has been previously reported (Manchado et al., 2008) and our observations of changes in gene transcripts compliments the increased size of thyroglobulin rich follicles observed by histology in sole (Delgado et al., 2006). In addition, to the synthetic machinery for production of THs, carrier proteins like transthyretin (*ttr*) and cellular membrane transporters such as *mct8* and *slc75a* (Arjona et al., 2011; Connors et al., 2010; Friesema et al., 2005; Heuer and Visser, 2013; Van Der Deure et al., 2010) were also differentially regulated. The *ttr* gene was up regulated across sole metamorphosis, *mct8* was upregulated around the metamorphic climax, and the *slc7a5* gene was abundant and stably expressed from pre-metamorphosis onwards. The presence of binding proteins for hormone circulation and cellular transporters contributes to efficient TH distribution and cellular uptake and action in responsive cells.

The local regulation of TH function is fine-tuned by deiodinases, which are crucial for the timing of metamorphic events in amphibians and flatfishes (Alves et al., 2017; Darras et al., 2015; Isorna et al., 2009; Manchado et al., 2009; Marchand et al., 2004). Deiodinase expression dynamics during fish metamorphosis are species-specific (Becker et al., 1997; Darras et al., 2015; Heijlen et al., 2014; Isorna et al., 2009; Liu and Chan, 2002; Walpita et al., 2007). For example, in sea bream, *dio1* and *dio2* expression peaked at the metamorphic climax, then declined, while *dio3* expression remained constant (Campinho et al., 2010). In the flatfish turbot, only *dio3* expression was upregulated (Marchand et al., 2004). Interestingly the present transcriptome results in sole were aligned with those of olive flounder (Itoh et al., 2010), where *dio1* expression was significantly modified and is important for metamorphosis. Previous studies in Senegalese sole suggested a decline in *dio3* expression during mid-late metamorphosis, while *dio2* expression remained stable (Isorna et al., 2009). In contrast, we found that both *dio1* gene isoforms were expressed during metamorphosis, with one isoform upregulated from the onset. This discrepancy may be due to differences in approach and the increased sensitivity of methods and sequence annotation in recent years.

Four TR isoforms (*traA*, *traB*, *trb1*, *trb2*) were expressed during metamorphosis as previously reported in Senegalese sole (Isorna et al., 2009; Manchado et al., 2009) and other fish species (Galay-Burgos et al., 2008; Harada et al., 2008; Kawakami et al., 2003a, 2003b; Marchand et al., 2001; Yamano et al., 1994). In sole, *traB* was highly and continuously expressed, a pattern that differs from their T4 content, while *trb2* was upregulated suggesting it has a central role in Senegalese sole metamorphosis, as previously reported by Manchado et al. (2009). *TraA* was also upregulated during metamorphosis. The dynamics of TR expression during sole metamorphosis mirrored the patterns observed in other flatfish (Galay-Burgos et al., 2008; Isorna et al., 2009; Oriane Marchand et al., 2004; Yamano and Inui, 1995; Yamano and Miwa, 1998). The cellular actions of THs also depend on co-repressors and co-activators (Harvey and Williams, 2002; Torchia et al., 1998; Yen, 2001; Zhang and Lazar, 2000) and in sole larvae, *n-cor1* and *n-coa1* gene expression was stable from D7 (7 dph), and *n-coa2* was upregulated during metamorphosis. The coordinated expression of *tr* and *rxr* genes



Thyroid Function	
Thyroid Follicles Formation & HPT-axis Regulators	*pax8 (1); trh-r (1); trh-de (1)*

Immune Response	
B Lymphocytes	*ebf (1)*
T Lymphocytes	*notch1b (1)*
Innate Lymphoid Cells	*gata-3 (1)*

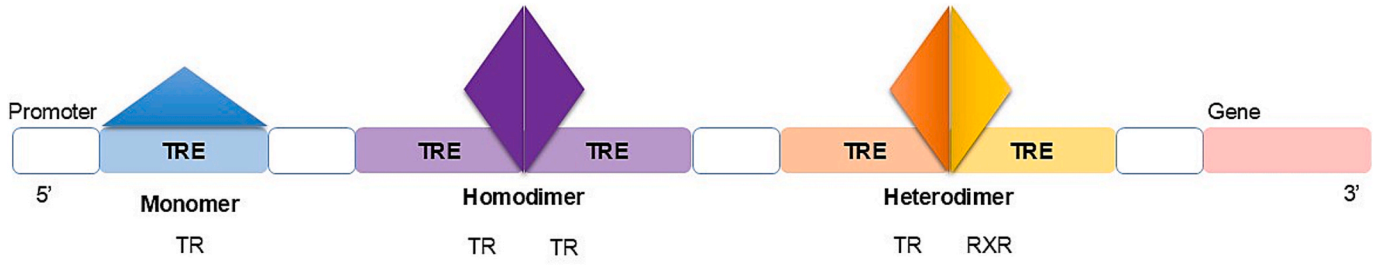
Thyroid Function	
TH Synthesis	*tg (1)*
TH Deiodination & Action	*dio1 (1); trαA (1)*

Immune Response	
Hematopoietic Stem Cell Markers	*cd34 (1)*
Mast Cells	*cpa5 (1)*
T Lymphocytes	*tcf7-like (1); bcl11b (1)*
Cytokines	*tgfβ1-like (1)*
Acute Phase Proteins	*fibrinogen-like (1)*

Thyroid Function	
TH Action	*n-cor2 (1)*

Immune Response	
Macrophage & Dendritic Cells	*scar (3); nlr (1)*
B Lymphocytes	*e2a (2); notch2 (1)*
Somatic Recombination	*rbpj-a (1); rbpj-like (1)*
Natural Killer Cells	*stat5 (1); foxo1 (1)*
Cytokines	*tgfβ2 (1)*
Acute Phase Proteins	*ftl-like (1); α2m (1)*
Complement System	*c1ql3 (1); c6 (1)*

Fig. 7. (*continued*).



Receptor	TRE sequence
TRα	*ntgnGntCacan*
	nnntGgtCannn
TRβ	*nnntGgtCannn*

Receptor	DNA sequence
RXRβ	*gtcAgTgac*
	nnnagGtCatnnn

Fig. 8. Schematic representation of putative binding modes of thyroid receptors (TRs) to thyroid response elements (TREs) in the promoter region of TH-responsive genes: monomers (TR), homodimers (TR-TR), or heterodimers with the retinoid X receptor (TR-RXR). The accompanying tables detail the putative consensus sequences for TR and RXR binding sites. Upper case red letters indicate positions with a high consensus value (90 %), while lower case blue letters indicate positions with a low consensus value (50 %). *n* represents any nucleotide. The sequence, frame orientation, and relative location to the transcription start site of the TR and RXR binding sites are summarized in Supplementary Table S16. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

during sole development supports their previously reported role in fine-tuning the timing of tissue development (Shi et al., 1994; Wang and Brown, 1993; Wong and Shi, 1995; Yaoita and Brown, 1990).

Our study provides support for TH involvement in immune system maturation during Senegalese sole larval metamorphosis. HPT axis-related genes clustered with immune function-related genes that contained putative TREs in their promoters. In sole larvae, *tgb*, *dio1*, and *trαA* gene expression patterns were matched by genes of the defence response and cytokine activity. Mammalian studies show that cells of innate defence metabolize THs via deiodinases and use TRαA to

modulate the expression of genes combating infection and inflammation (Alamino et al., 2015; Boelen et al., 2005; Boelen et al., 2009; Chen et al., 2012; Diano and Horvath, 2008; Kwakkel et al., 2014; Mascanfroni et al., 2008; van der Spek et al., 2018; Van Der Spek et al., 2016). In this context our results are intriguing since they suggest that the TH driven metamorphosis in sole is associated with overt activation of lymphoid organ development, particularly the thymus. *Trb2* expression in sole during metamorphosis matched expression of dendritic cell markers, mirroring observations in mammalian dendritic cells where *trb* modulates TH-dependent gene expression (Mascanfroni et al., 2010; Montesinos et al., 2012). Additionally, *trb2* was correlated with self-antigen processing and presentation via MHC I, T cell differentiation, somatic recombination, natural killer cell function, and the complement system. Overall, the clustering approach used for gene expression pattern analyses in sole larvae suggests a link between THs and various aspects of organ functionality, lineage maturation, and tissue specificity of the immune system, which is consistent with existing knowledge from mammalian species.

The humoral response, proposed as the main immune response in fish larvae, involves soluble molecules such as chemokines, cytokines, antimicrobial peptides, acute-phase proteins, and complement system elements (Costas et al., 2013; Ellis, 1999). Rainbow trout (Huttenhuis et al., 2006), common carp (Wang et al., 2011), sea bream (Li et al., 2021; Mulero et al., 2008), Atlantic cod (Lanes et al., 2012; Magnadóttir et al., 2018), and halibut (Magnadottir et al., 2019) all express these molecules upon hatching, enabling action of innate immune defence mechanisms (Bjørgen, 2022; Mulero et al., 2008). Our results showed that pre-metamorphic Senegalese sole larvae at D7 (7 dph) expressed several innate humoral factor genes, including β-defensins, hepcidins, serum amyloid A, ferritin, mannose-binding lectin, complement components (*c1* and *c4*), interleukins (*il1b*, *il15*, *il11*, and *il12*), and chemokines (*ccl19*, *ccl25*, and *ccl20*). The expression of these humoral immunity factors prior to substantial changes in TH levels suggests they are not dependent on THs. In contrast, other genes involved in the complement pathway (*c3a*, *c3b*, and membrane attack complex components *c5*, *c6*, *c8a*, and *c9*), chemokines (*cxcl12* and *cxcl14*), and acute-phase proteins (C-reactive protein, transferrin, lysozyme, alpha-2-macroglobulin, fibrinogen, and thrombin) were all up-regulated during metamorphosis and belonged to gene clusters shared by the HPT axis and immune-related genes.

Considering acquired immunity both humoral and cellular elements and lymphatic tissue were considered during metamorphosis. In Senegalese sole, the kidney develops with excretory functions at hatching (Howell et al., 2019). The transcriptome results indicate that immune-related genes such as *pax5*, involved in B-cell activation (Hagman and Lukin, 2007; Roessler et al., 2007; Schebesta et al., 2007; Wu et al., 2019; Zwollo, 2011; Zwollo et al., 2008), and *cdc42*, associated with B-cell development (Burbage et al., 2015), were downregulated by 12 dph (S0), suggesting that the kidney may already have immune-related activity before metamorphosis. Previous studies in marine teleosts like turbot, salmon, sea bass, and catfish revealed early lymphoid organ development, with the spleen forming around 4 dph (Dos Santos et al., 2000; Langenau et al., 2002; Padrós et al., 2011; Petrie-Hanson and Ainsworth, 2000; Zapata et al., 2006). Our sole transcriptome results suggest that the thymus develops during metamorphosis, and key genes for this process such as *cdca7a*, *fam49al*, *mcm2*, *med24*, *nrp1a*, *tbl3*, and *wdr55*, were only downregulated post-metamorphosis (Fig. 4e, Supplementary Table S9), as has previously been reported in the rainbow trout (Padrós et al. (2011).

In general B cell development occurs earlier in freshwater species than marine species, although maturation timelines vary by species. In rainbow trout, B lymphocytes appear before hatching (Castillo et al., 1993) and rapidly acquire the ability to express immunoglobulins (Ig) (Bjørgen et al., 2022; Razquin et al., 1990). In marine species, like the European seabass they express IgM 12 dph and the lymphoid kidney is fully developed by 50 dph (Breuil et al., 1997), while in the Atlantic halibut the kidney develops at hatching, but IgM-positive cells only appear at 66 dph (Patel et al., 2009). In our sole transcriptome B cell-specific biomarkers, such as *ebf*, *pax5*, and *c-myb*, were down-regulated during metamorphosis, while others, like *e2a* and *notch2*, were upregulated. These results suggest that the B cells had already started to develop before metamorphosis. T cells also seem to develop earlier in freshwater species compared to marine species and in rainbow trout (Bjørgen et al., 2022) and common carp (Botham and Manning, 1981), the thymus is lymphopoietic a few days post-hatching. While in the marine, European sea bass, T cells were identified at around 25 dph, but T cell receptor gene expression was not detected until 73 dph (Scapigliati et al., 1995). In the sole, T cell differentiation occurred during metamorphosis as indicated by the upregulation of DE biomarkers such as *cd95*, *runx3*, *tcf7*, *bcl11b*, *thpoka*, and *cd45ra*. Furthermore, the occurrence of somatic recombination, essential for B and T cell receptor formation, was inferred by the upregulation of genes such as *rag1* and *rbpj* (Huttenhuis et al., 2005) during sole metamorphosis. In early developmental stages of the common carp and zebrafish, *rag* gene expression was reported much earlier, at 4 dpf (days post fertilization) in the thymus and pancreas, and 6 dpf in the kidney (Botham and Manning, 1981; Lam et al., 2004; Wang and Han, 2013; Willett et al., 1999).

TRE sequence motifs and their location within the promoter region are largely unknown in fish (Gagne et al., 2013) and this study is the first to summarize nucleotide sequences of TREs in teleost fish. TRE motif position and the consensus sequence is conserved between mammals (mouse and human) (Yamada et al., 1990; Zhang et al., 1998). Consensus TRE binding site sequences (PROMO results) in DE immune genes or genes that clustered with HPT-axis genes during metamorphosis were TRα-specific (*n*tg*n*G*n*tCaca*n*) or bound both TR types (α and β, *nnn*tGgtCa*nnn*). The sole TRα-specific or TRα and TRβ-specific binding TREs shared 55.6 % and 71.4 % identity, respectively with classical TR binding half-sites (AGGTCA) in mammals (Baniahmad et al., 1990; Brent et al., 1992; Farsetti et al., 1992; Katz and Koenig, 1994; Koenig et al., 1987). Variability in TRE sequences is proposed to create flexibility in transcription factor binding (Dong et al., 2007; Gagne et al., 2013) and the multiple TREs in Senegalese sole may explain the broad suite of activities of THs during its metamorphic transformation.

Previous research in humans and rodents identified TH-responsive genes, with TREs in HPT axis-related genes (*trh*, *dio1*, *dio3*) (Alves et al., 2017; Satoh et al., 1999; Zhang et al., 1998) and immune-related genes (*nr4a1*) (Dong et al., 2007). In sole, core immune pathway-related transcripts, such as *cacna1fb*, *kcnj11l*, *smarcd1*, and *efna2a*, possessed multiple and diverse TRE motifs, indicating a greater potential for regulation by THs. Other transcripts like *cish2*, *crhb*, *gata3*, *pbx1a*, *rac2*, *irf7*, *gpr1*, and *enpp1* had a unique pan-TR binding sequence in their promoters, which may suggest a more flexible responsiveness to THs. Finally, some immune-related transcripts, including *cxcl12*, *csf1r*, *pglyrp6*, *rag1*, *C3a.1*, *C3a.4*, and *C2*, lacked TR binding sites and are probably not TH regulated. The results of our study revealed a novel and important link between TH-regulated immune development and metamorphosis, but further studies are needed to confirm the TH-responsiveness of TRE containing immune-related genes and to distinguish between positive and negative TREs. Non-genomic effects of THs (Sterling et al., 1978; Sterling et al., 1980; Sterling and Brenner, 1995) also need to be considered.

5. Conclusion

The global gene expression patterns during metamorphosis of the Senegalese sole, confirmed the outcome of previous studies highlighting the central importance of the thyroid axis. By clustering gene transcript expression across metamorphosis with HPT-axis genes a series of immune-related gene transcripts were identified. Metamorphosis related changes inferred from DE genes were linked to thymus development, wound healing, granulocyte migration, the cytokine response, maturation of macrophages, dendritic cells, natural killer cells, and T

lymphocytes. We note that THs are unlikely to be mandatory for all aspects of immune system development during metamorphosis in the sole. Nevertheless, clustering analysis identified numerous immune-related transcripts that are candidates for TH regulation, which was confirmed by the identification of TR binding sites in the promoter of 84 out of the 133 immune-related DE genes identified. TREs varied in abundance and putative binding affinity to TRα or TRβ, and we propose that this increases the flexibility of TH regulatory processes during sole metamorphosis.

CRediT authorship contribution statement

Sandra C. Silva: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Teresa M. Correia:** Writing – review & editing, Supervision. **Manuel Manchado:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Deborah M. Power:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2025.114755.

Data availability

Data will be made available on request.

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