Expression of immune genes during metamorphosis of *Xenopus*: a survey

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TABLE OF CONTENTS

1. ABSTRACT

This review focuses on what is known about the immune transcriptome during metamorphosis of *Xenopus laevis* and *Silurana (Xenopus) tropicalis*. This subject is of importance to obtain a global understanding of the physiological changes operating during metamorphosis. In turn, a good knowledge of the physiology of amphibian metamorphosis may contribute to the fight against amphibian decline and help the development of alternative toxicologic assays. By examining what is known on the expression of innate and adaptive immune genes during metamorphosis, it becomes clear that our knowledge of the anatomy of the tadpole "immunome" is fragmentary. Since a wealth of data sits in cDNA sequences, I am making a first attempt to enrich our knowledge on this subject. I exemplify that mining EST data can rapidly provide us with the necessary tools to unravel the cross-talk between thyroid hormone signalling during metamorphosis and larval immune system changes.

2. INTRODUCTION

Animal metamorphosis is a biological process raising several issues in the fields of evolution, developmental biology and molecular genetics. Metamorphosis is defined as the process by which a larva transforms into an imago resembling the adult, the latter being the reproductive form of a given species. This transformation is accompanied by morphological changes and ecological transitions. Descriptions and explanations of the morphological and biochemical changes occuring during amphibian anuran metamorphosis are numerous (1-3, Figure 1). An important but often neglected aspect of metamorphosis is the requirement for the tadpole to be able to cross an immune barrier before becoming an imago. As a consequence studies linking metamorphosis and the amphibian immune system are lagging behind.

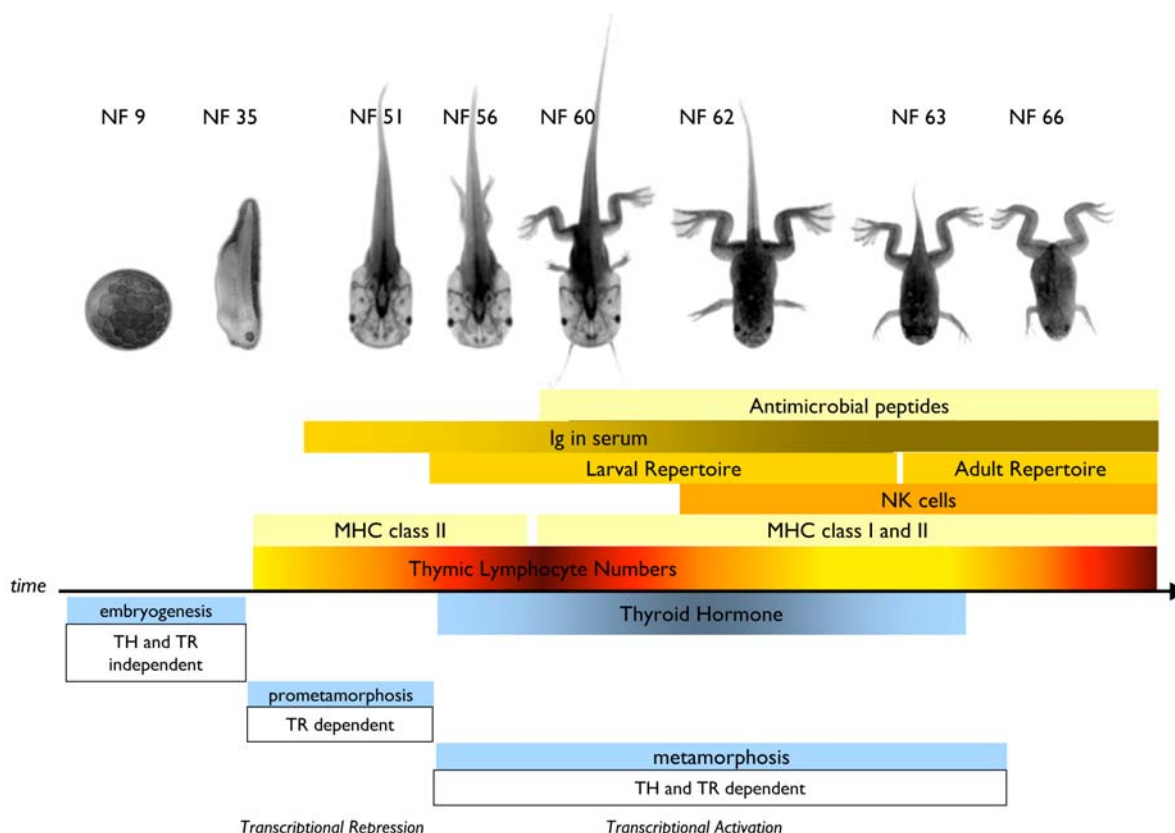This review focuses on what is known about the

Figure 1. *Xenopus* metamorphosis and the immune system. This figure depicts the timing of appearance of immune functions during *Xenopus* metamorphosis. Regarding thymic lymphocyte numbers, the yellow-red colour represent low-high cell numbers, respectively. Thyroid hormone plasmatic levels are represented in shades of blue, proportional to the concentration. Adapted with permission from 6.

expression patterns of immune genes (the immune transcriptome) during metamorphosis of *Xenopus laevis* and *Xenopus tropicalis*. I refer the reader to L. Rollins-Smith (this issue) for more information on the biology of the larval immune system. I will present some reasons why it is of importance to increase our knowledge on immune gene expression during metamorphosis, and what is so special about frog metamorphosis and the immune system. I will briefly remind the reader of the current knowledge on the endocrine control of metamorphosis. A description of what is known so far on the expression of innate and adaptive immune gene expression during metamorphosis will follow. Finally, I will review what we can gather from mining available genomic data in *Xenopus* and how this can be useful to rapidly increase our knowledge on the regulation of the expression of immune genes during this post-embryonic developmental process.

3. AMPHIBIAN METAMORPHOSIS AND THE IMMUNE SYSTEM

3.1. Why study the expression of immune genes during *Xenopus* metamorphosis?

In recent years, amphibian decline in the wild has become a global concern (4 and http://amphibiaweb.org/).

Chemical pollutants (in particular pesticides) acting on detoxification, endocrine and immune systems are some of the causes of this decline. Amphibian pathogens are another possible cause of decline. Since the completion of metamorphosis is complex and relies on the neuroendocrine and immune systems, it is likely that amphibians are extremely sensitive to pollutants. A better understanding of the regulation of the expression of immune genes during metamorphosis will certainly be beneficial to toxicological studies.

A *Xenopus* embryo hatches after two days of an embryonic phase of development and under the protection of the chorion. Since the development of *Xenopus* is external, the newly hatched tadpole will be in direct contact with environmental pathogens. Therefore, the first task of the immune system will be to develop rapidly to cope with this situation. Another task of the immune system will be to enable the replacement of larval-type cells by the adult-type in essentially all the tissues but not at the same time during metamorphosis. An additional problem here is to educate the immune system to tolerate all the new adult-type gene products that start to be expressed at the time of metamorphosis. A final task of the immune system will be

to perform well in the context of the new ecological niche of the imago and adult forms.

Moreover, the amphibian model lies at a critical node in the vertebrate phylogeny. The last common ancestor between mammals and amphibians lived about 370 million years ago. Such an evolutionary distance enables insightful comparative immunogenetic studies (5). Since the 1950s, the preferred amphibian reference species among immunologists and biologists has gradually become the anuran *Xenopus* frog species. Most studies on metamorphosis purpose the amphibian anuran model *Xenopus laevis*. However, the *X. laevis* genome is pseudotetraploid and it is common to find one gene represented by two paralogous loci ("allogenes") in *X. laevis* (see the case of the FcR-like receptors considered by Guselnikov *et al.* in this issue). Furthermore, nucleotide sequence similarity between "allogenes" is variable and there is no genomic sequence to help decipher a global view. This feature of *X. laevis* genome implies that when highly similar "allogene" expression differs in time or space, molecular genetics experiments are complicated and their results are affected. On a genomic scale, and for immunogenetics such a technical limitation has important consequences. Fortunately, some immune genes are functionally diploid in *X. laevis* (6). *X. tropicalis,* also named *Silurana tropicalis,* is an alternative model for such genetic studies. With more than a million of ESTs and the genome sequence availability, large-scale surveys of gene expression are easier to tackle and interpret in this diploid anuran.

3.2. What is so special about metamorphosis and the immune system?

The immune system of the tadpole is not equivalent to the immune system found in adults (7). Differences in antibody responses and allograft rejection are observed between larvae and adults. A novel antibody repertoire is formed upon metamorphosis completion and tadpoles are unable to reject grafts differing only by minor histocompatibility antigens unlike adults (8).

The major organs of the larval immune system are the thymus, the spleen, the lymph glands, the pronephros and the liver (9). In *Xenopus*, lymph glands are represented by pairs of four ventral and two dorsal lymphoid cavity bodies found in the branchial region, and scattered lympho-epithelial tissues found along the alimentary tract. The lymph glands and the pronephros are lost during metamorphosis while thymus, spleen and liver persist without gross morphological changes. However, metamorphosis is associated with an important turnover of the lymphocyte population with a decline in the number of larval lymphocytes by ~ 40 %. The ventral displacement of the thymic glands during metamorphosis is associated with massive (90 to 95 %) thymocytes apoptosis. Just at the end of metamorphosis a new wave of stem cells migration occurs followed by new histogenesis. Some larval memory B and T cells persist through metamorphosis and are able to elicit a classical accelerated antibody response to antigen in adults (8). Lymphopoiesis in late larvae before or after

metamorphosis proceeds from the same population of stem cells (10). Remarkably, the contribution from ventral blood islands and dorsal stem cell compartments is cyclic (11).

Some authors proposed a theory in which the immune system would be largely involved in the specific elimination of tadpole larval cells during metamorphosis (12 and see also in this issue). This line of research is interesting in that it provides a systemic approach to metamorphosis, complementary to the current dogma on anuran metamorphosis in which the thyroid axis is the sole factor under study.

4. NEUROENDOCRINE CONTROL OF METAMORPHOSIS

We know that hormones control the numerous morphological and physiological changes that occur during metamorphosis (1-3). In 1912, Gudernatsch did an experiment in which young tadpoles fed with thyroid gland extracts engaged precociously in metamorphosis. These results evidenced the presence in the thyroid gland of a factor playing a critical role in triggering metamorphosis. The molecular nature of this factor was determined later: it is 3,5,3',5' tetraiodothyronine, also known as T4, thyroxin or thyroid hormone (TH). Numerous experiments confirmed that TH (as T4 or T3) is sufficient but also necessary for the completion of metamorphosis. A chemical thyroidectomy (using potassium perchlorate for example) or a surgical one leads to a sustained growth of tadpoles without metamorphosis. Other hormones such as corticoids (13) are known to play important roles as well, affecting the speed of metamorphosis. Prolactin has been shown to inhibit TH signalling at the level of its receptor (14).

When the immune system is challenged, glucocorticoids control the cytokine response (see Kinney and Cohen, this issue). Conversely, cytokines modulate glucocorticoid production by the hypothalamic-pituitary-adrenal axis. Thus, the immunological status of the tadpole affects the completion of metamorphosis on a systemic level. There is a more direct mechanistic influence of the immune functions on the changes of cell fates at least regarding the skin (15).

We know that TH signalling is interpreted at the cellular level by transcriptional regulations as thyroid hormone receptors encode transcription factors (1,3). Moreover, it has been established that during the post-embryonic period of development, thyroid hormone (TH) acts by reprogramming cell fate. The very nature of this reprogrammation depends on both positional cues and on cell type. During metamorphosis, some cells will start a programmed cell death by apoptosis while others will actively proliferate and differentiate. This is a clear evidence that TH operates a non-specific stimulus and by itself is not a carrier of instructions to the tissues: targeted cells must carry the information of their fate upon metamorphosis within themselves.

The regulation of gene transcription during metamorphosis is an important area of research. The most dramatic changes of gene expression during metamorphosis have been documented such as the switches of hemoglobin, albumin and the transition from ammonotelism to ureotelism in the liver (2). Differential screens have been performed starting with mRNAs from brain, limbs, intestine or tail taken at different metamorphic stages (3,16-17). These experiments have been performed in the context of induced metamorphosis in different amphibian species and have led to the identification of so-called TH target genes. About twenty genes in each tissue displayed differential expression. These genes were grouped in early and late-response categories according to their timing of responsiveness to TH and encode proteins associated with a variety of molecular functions (transcription factors, enzymes, structural proteins...). A few players were identified as being part of the transcriptional cascade triggering apoptosis. Altogether, these findings do not provide us with a global picture of the physiologically relevant transcriptional changes. More global studies were deemed necessary to study these aspects of metamorphosis. The use of macroarrays (400 cDNA clones of *X. laevis* and *R. catesbeiana*, (18-19) and of microarrays (Agilent *X. laevis* DNA microarray, 20) enabled the identification of additional target genes, but failed short of a genomic overview. In all these studies, the regulation of immune genes by TH was neglected.

5. WHAT WE KNOW SO FAR

We are lacking knowledge about regulation of the immune genes during metamorphosis. In an excellent recent book on the subject of amphibian metamorphosis (3), only one sentence refers to the changes of the immune system during the tadpole transformation into an adult (p48 in 3). The last review on the subject was published in 1998 and comes from Louise Rollins-Smith (7).

5.1. Innate immunity gene expression
5.1.1. Antimicrobial peptides

Antimicrobial peptides constitute an important first line of defence provided by the skin against pathogens (21). Although the literature on the sequence and activity of amphibian antimicrobial peptides is abundant (see 22 for a review), we have little genomic data on the corresponding *Xenopus* genes (23-24). The inspection of the *X. tropicalis* genome reveals at least seven transcription units encoding antimicrobial peptides that are clustered over 350 kbp on scaffold_811 without intervening gene. Each transcription unit is composed of four to five exons as deduced from EST alignments, and expressed from the onset of metamorphosis, in skin, bone and thymus. Remarkably, all these transcription units share a highly conserved amino-terminal portion (including the signal peptide) with cholecystokinin and gastrin neuropeptides, encoded by a single exon (25). However, the genes encoding cholecystokinin (scaffold_1166), xenoxin (scaffold_521) and TRH (scaffold_353) are located on separate scaffolds, and in syntenic regions compared to mammalian genomes. The structure of this locus for antimicrobial peptides

reflects the combinatorial rearrangements of exons that occurred during evolution (25). It is tempting to propose that splicing events may produce a combination of mRNA for different preproproteins encoding a variety of peptides and playing important roles for innate immunity.

We still know little on the expression of antimicrobial peptide genes during ontogenesis. Transcripts encoding these peptides are invariably found in cells of the granular glands in adult skin (26), and sometimes in the mucosa of the gastrointestinal system (27). A single article describes the expression of antimicrobial peptides during metamorphosis. The expression of magainin and PGLa mRNAs during development correlates with the metamorphic changes, and is induced upon TH treatment (28). This finding is corroborated by studies on the development of granular glands. These granular glands in amphibians are known to secrete antimicrobial peptides, neuromuscular toxins (e.g. xenoxins) and neuropeptides (e.g. thyrotropin releasing hormone, 29). In *Xenopus*, few granular glands rudiments containing caerulein peptides are found on the dorsal skin of Niewkoop and Faber stage 57 tadpoles (30). From stage 58 to 60, the number of functional gland increases. Experiments in which skin explants were cultivated in serum with or without TH showed that such an increase of functional granular glands cannot be explained by the sole effect of TH stimulation (30).

In conclusion, the larval skin seems to be devoid of granular glands able to synthesize and secrete antimicrobial peptides. There is no evidence regarding the expression of antimicrobial peptides during pre and pro-metamorphic stages. Afterward, these glands will develop rapidly and become functional at the climax of metamorphosis. The situation in the gastrointestinal system is currently unknown and awaits further investigation.

5.1.2. Natural Killer cells and their receptors

Natural killer cells are known to play a critical role in innate immune response by destroying cells that do not express MHC class I protein such as tumors or virus infected cells. Since the tadpole does not express MHC class I before late metamorphosis, it is of interest to know if, when and how NK cells do differentiate in such an organism.

The definitive identification of *bona fide* NK cells in amphibians is relatively recent (31-32). The ontogeny of these cells was studied using the 1F8 antibody in *X. laevis*. There is no 1F8 positive cells detected in larval splenocytes at pro-metamorphic stage 54-55 and just a few at stage 56-58 (32). The conclusion that can be made is that NK cells in late tadpoles either express low-levels of the antigen recognized by the 1F8 antibody or are not fully mature. Another possibility, difficult to test at this time, is that tadpoles have a distinct type of NK cells that is 1F8 negative. These findings are consistent with the theory in which MHC class I positive cells are essential for NK cells education and self-tolerance development. These NK cells are unlikely to play a role in the tadpole immune system.

Activating and inhibiting receptors controlling NK cell activity may be expressed during larval stages of development. However, we know very little on this subject at the present time since the homologues of C-type lectin receptors, Fc receptors, KIR like molecules await further identification and characterization (see Taranin *et al.*, this issue).

5.2. Acquired immunity gene expression
5.2.1 B and T-cell ontogeny

A larval and an adult immune system can be distinguished based on several criterias including the number and the distribution of lymphocytes in the organism (7-8). The transition between these two immune systems is characterized by a drastic 40% decrease in the number of lymphocytes populating the liver, the spleen and more than 90% thymocyte loss in the thymus during metamorphosis (Figure 1). Importantly, some larval lymphocytes persist through adulthood (7).

What we know on B-cell ontogeny in *Xenopus* has been reviewed seven years ago and our current view is represented on figure 1 (8). There is a difference of B cell repertoire between the tadpole and the adult. Tadpole antibodies are less heterogeneous and of lower affinity (8). The IgM to IgY switch is inefficient in the tadpole. Additional Ig isotypes (IgD, IgF) were discovered recently but their expression during larval life has not been documented yet (5,33-34). In a nutshell, larval and adult B cells can be distinguished according to their capping properties and their distribution in the organism, but nothing yet is known on their differences in terms of gene expression profiles.

T-cell receptor genes for alpha, beta, and gamma subunits have been cloned in *X. laevis* and their diversity in the adult has been characterized (7, 35-37). Much like immunoglobulin genes, TCR gene organization and diversity appears well conserved in *Xenopus*. Diversity of V alpha genes is higher than V gamma. An in-depth analysis of *X. tropicalis* genome sequences should give more details on the extent and variability among TCR genes in this species.

The activity of B-cells depends on RAG proteins mediated recombination of the immunoglobulin genes. RAG mRNA are first detected at tailbud stages but RAG enzyme activity is down-regulated during metamorphosis (11). In the adult both RAG1 and RAG2 are preferentially expressed in the thymus, but transcripts are also found as well in the liver, spleen and even kidney. Alternative RAG2 transcripts are found in oocytes and testis (38). B cell function also depends on Activation Induced Deaminase, whose expression is documented in larvae and adult (39).

5.2.2. MHC and metamorphosis

The regulation of MHC gene expression during metamorphosis has been described twenty years ago. MHC class I molecules are not found at the cell surface of most tissues of immunocompetent tadpoles before metamorphosis (40). Thus the tadpole immune system development and function appears not to require class I expression. Flajnik and collaborators made an interesting observation when they first described the expression of MHC genes during metamorphosis: MHC class I antigen could be detected on the surface of splenocytes from tadpoles three months after the inhibition of metamorphosis with perchlorate (40). This observation support the existence of a regulatory mechanism governing the expression of MHC class I genes independently of the thyroid axis.

B cells, macrophages, spleen reticulum, thymus epithelium and cells from the oral cavity of the tadpole express MHC class II molecules. However, larval T-cells are class II negative whereas B cells are positive. Such a widespread distribution of class II molecules in a variety of larval epithelial cells may be used to present conventional antigens to T-cells.

During metamorphosis, all lymphocytes, including T cells, start expressing class II molecules on their surface, while MHC class I become ubiquitously expressed (41 and see Figure 1). The differential expression of MHC before and after metamorphosis is likely to account for the lack of autoimmunity during tadpole transformation.

5.2.3. Hormonal regulations

Hypophysectomized and perchlorate-treated tadpoles have been instrumental in the investigations of the endocrine regulations of the immune system ontogeny in *Xenopus*. The absence of pituitary hormones does not impair liver hematopoietic stem cells development but leads to a limited proliferation of lymphocyte populations in the spleen and thymus (42). The natural increase of corticosteroid hormones during metamorphosis has been found to play a major role in the drastic decrease of lymphocytes population by apoptosis. In contrast, TH was not found to affect directly this event (7).

6. WHAT WE CAN GATHER FROM MINING GENOMIC DATA

With 1,271,375 and 677,784 ESTs submitted to Genbank (August 24, 2007) *X. tropicalis* and *X. laevis* transcriptomes have been sampled to a good extent using partial cDNA sequencing (43). Analysis can be made on libraries derived from organs that are known to be the sites of immune cells maturation in *Xenopus*, i.e. liver, spleen and thymus.

In *X. tropicalis,* three cDNA libraries made from lymphoreticular organs (adult thymus and spleen), and one library made from adult liver, have been subjected to cDNA sequencing (see Table 1). The depth of sampling for each of these libraries can be evaluated from the mean number of cDNA clone tags sequenced and is in the order of ~10,000 clones/library. This sampling value is similar

Expression of immune genes during metamorphosis of *Xenopus*: a survey

Table 1. *Xenopus* cDNA resources for the study of immune transcriptomes

Species	cDNA library	Vector	Description	XGC FL	Nb clones with ESTs	Nb total ESTs	Nb ESTs in UniGene	Nb UniGene clusters
X. tropicalis	NIH_XGC_tropSpl1	pCS107	Adult spleen fraction > 1000 nt	41	9 729	19 050	16 065	4 542
X. tropicalis	NICHD_XGC_tropSp1	pCS107	Adult spleen	13	9 192	17 415	16 703	3 584
X. tropicalis	NICHD_XGC_tropThy1	pCS107	Adult thymus	22	8 614	16 030	15 258	3 724
X. tropicalis	NIH_XGC_tropLiv1	pCS107	Adult liver fraction > 1000 nt	25	11 860	22 921	20 695	4 122
X. laevis	NICHD_XGC_Li1	pCMV-SPORT6	Adult liver	120	2 853	3 956	3 458	1 305
X. laevis	NICHD_XGC_Sp1	pCMV-SPORT6	Adult spleen	845	11 065	15 807	13 510	4 843
X. laevis	NICHD_XGC_sple_PHA	pExpress-1	Adult spleen PHA stimulated fraction > 1250 nt	73	4 403	5 465	4 775	2 234
X. laevis	NICHD_XGC_thy	pCS111	Adult thymus fraction > 1250 nt	70	4 408	5 862	5 060	2 239

from one library to another and reflected by the comparable numbers of corresponding UniGene clusters. Since these libraries have been significantly sampled and were not normalized, the inference of transcripts levels from EST counts is more likely to reflect the physiological conditions.

Similarly in *X. laevis,* three cDNA libraries made from lymphoreticular organs (adult thymus and spleen, with or without PHA stimulation) and one library made from adult liver have been sampled by sequencing (see Table 1). Here, we can observe that the number of cDNA clones sequenced per library varies from 2,853 (liver, see Table 1) to 11,065 (adult spleen). The depth of sampling is quite different from one library to the other. In this case, the inference of transcript levels from EST counts, even if it might be fruitful, is less likely to be a good indicator of the physiological reality.

This wealth of cDNA sequence data can enable to draw an inventory of genes expressed in lymphoreticular organs at larval or adult stage of development. It is of importance to remember that these expression data are rather crude and should serve only as a primer. Consequently, it is safe to analyze these EST expression data with conservative criteria to produce experimentally testable hypotheses. I asked elementary questions to these data sets: what are those transcripts found either specifically or differentially expressed in spleen-thymus-liver cDNA libraries ? Accordingly, I extracted UniGene clusters satisfying the following criteria: (i) the total number of ESTs for spleen/thymus/liver should exceed half the number of ESTs for the given UniGene cluster; (ii) the UniGene cluster is composed of ESTs derived from ten cDNA clones at least; (iii) the UniGene cluster is not made up only from liver derived cDNAs.

In *X. laevis*, 223 UniGene clusters were thus identified: 27 correspond to so-called transcribed locus because no similarities to any protein could be detected; 24 correspond to hypothetical (or orphan) proteins and 172 correspond to known proteins. In this last category, several genes known to play physiological roles in the immune function were found such as T cell receptors alpha and beta, MHC class I and II ; ROR gamma, T cell-specific transcription factor 1, etc (see Supplemental Table1 genes figured in bold). Evidence of expression at tadpole or metamorphic

stages was found for 147 (66%) of these UniGene clusters. Clustering these 147 genes based on their expression profile, I can observe five clusters (Figure 2). The red and orange clusters correspond to genes whose expression is found from early embryonic stage to metamorphosis and in adult spleen or thymus. In the red cluster, expression in adult tissues is stronger and the converse is observed in the orange cluster. This orange cluster is probably the less relevant in physiological terms. Chemokine is a remarkable feature of the red cluster with two chemokines and a receptor being expressed in this pattern. The orange cluster is the largest one with 49 genes. Genes expressed with a later onset during development are found in the yellow, green and blue clusters. A broad expression in adult tissues is observed for the yellow cluster and expression in adult spleen and thymus is more pronounced for genes in the green cluster. Overall, these three clusters contain numerous *bona fide* immune genes.

The yellow clusters groups together MHC class I and class II molecules, immunoglobulins, Wiskott-Aldrich syndrome, beta2-microglobulin and cathepsin-S. Interestingly, the same cluster contains the orphan gene12, identified in *Xenopus* as up-regulated in the tail by TH treatment. The inspection of the genes found in the green and blue cluster highlight that most genes active at metamorphosis stage are later involved in the adult immune system physiology. However, the time resolution of this analysis is limited and does not differentiate expression between early and late expression.

In *X. tropicalis*, 141 UniGene clusters were identified: 27 correspond to so-called transcribed locus; 20 correspond to orphan proteins and 94 correspond to known proteins. Similarly, immune genes are well represented (see Supplemental Table2 genes in bold). After comparing expression profiles, I find clusters with characteristics similar to those observed on X. laevis data (same colours on Figure 3). The exception is the small dark green cluster corresponding to genes expressed in the liver from tadpole stage.

The total number of gene expression profiles clustered in *X. tropicalis* is smaller than in *X. laevis* because of the lack of proper annotation of *X. tropicalis* immune genes. There is more information on MHC, immunoglobulins and T-cell receptor sequences in *X. laevis* than in *X. tropicalis*,
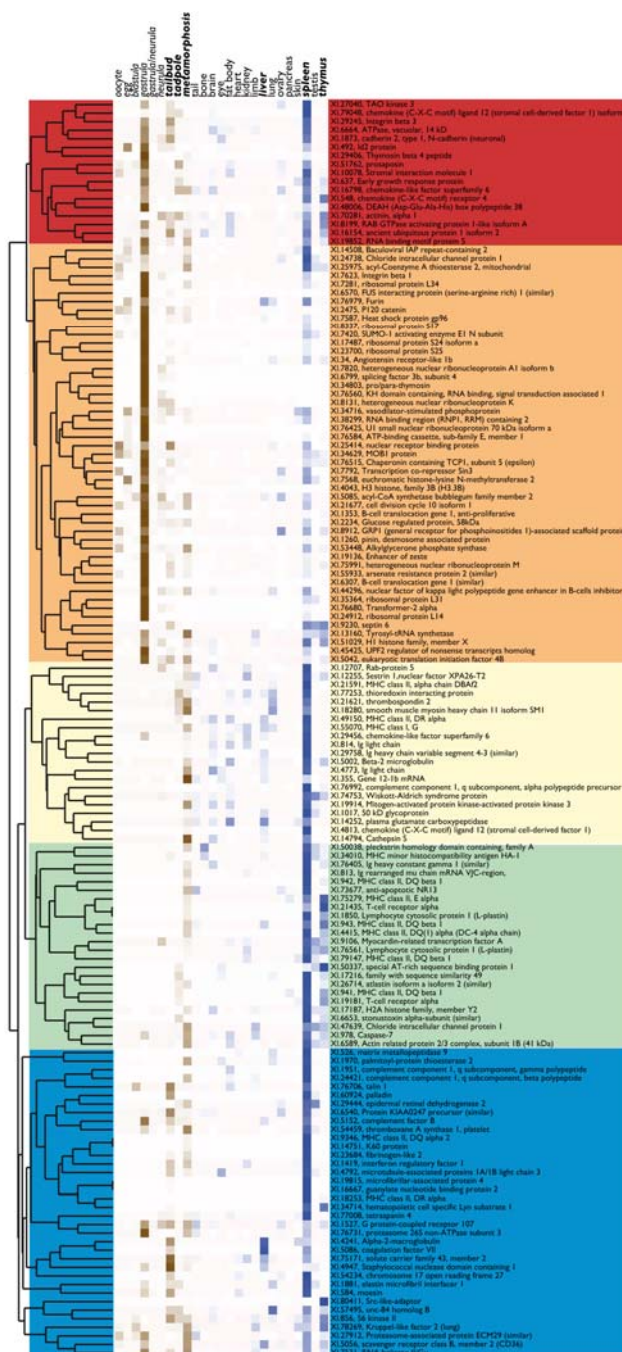
Figure 2. Digital expression profiles of *X. laevis* transcripts differentially expressed in lymphoreticular organs. Each line gives the expression profile of a given transcript represented by a UniGene cluster. The expression is deduced from counting the occurence of ESTs within a given cDNA library. The level of expression is colour coded in brown or blue shades, dark means evidence for high levels of transcripts and white means no evidence for expression. On the left, a cladogram depicts the similarity of expression profiles. Clusters are coloured according to their shared characteristic of expression. On the right, the cluster name and its annotation (i.e. the corresponding gene product description as deduced from sequence similarity analysis) are given. Each column corresponds to a category of stage of development (in brown) or adult tissue (blue). Note that a given category may correspond to several cDNA libraries. Here, only clusters for which evidence of differential expression were used to build the matrix of expression. Expression profiles derived from EST counts were analyzed using Cluster after centering and normalization of the data. The Spearman rank correlation metric was used for a complete linkage hierarchical clustering on the gene expression dimension. Optimization of the clustering was performed using FreeOView. Results were visualised using Java TreeView.
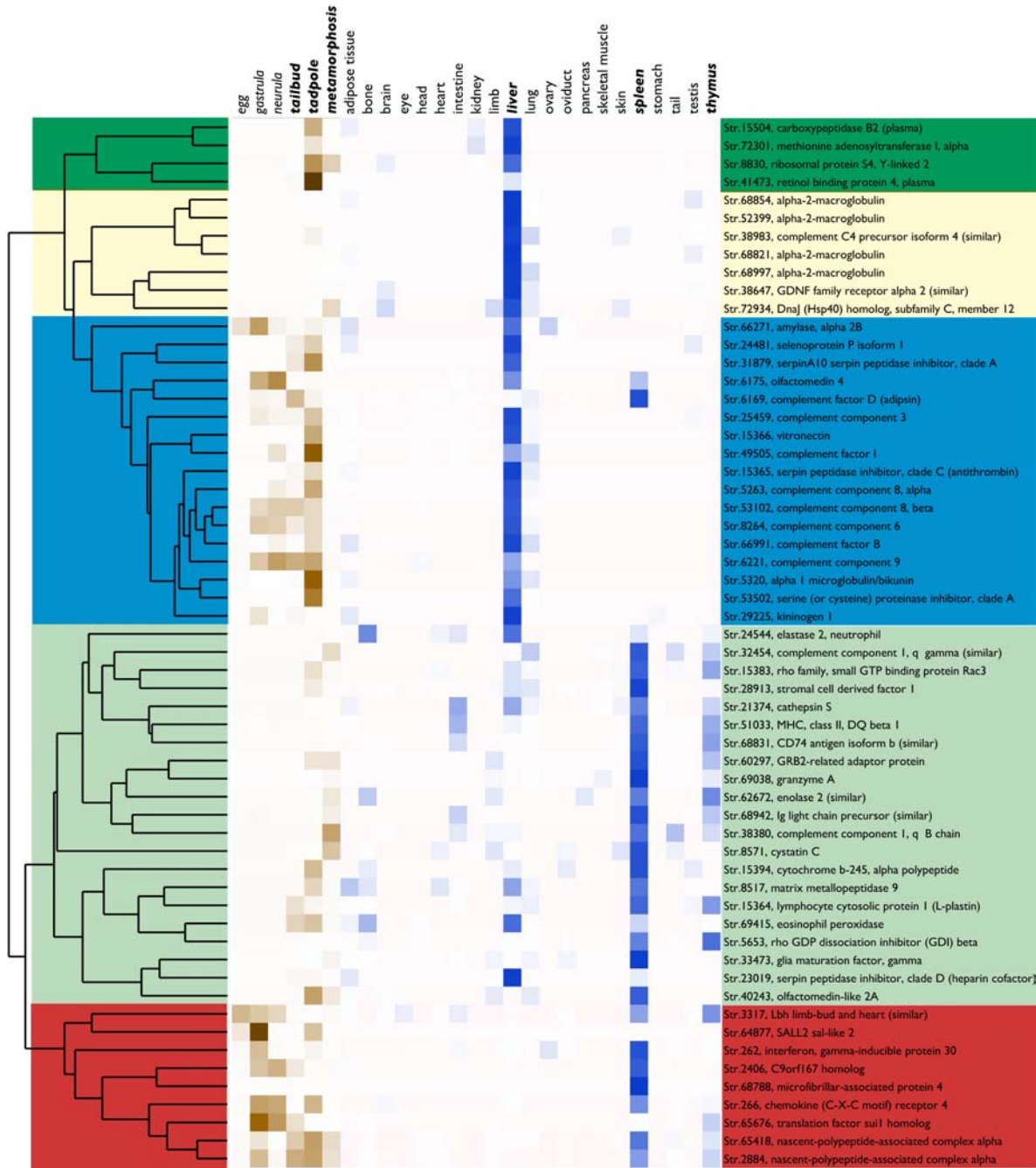
Figure 3. Digital expression profiles of *X. tropicalis* transcripts differentially expressed in lymphoreticular organs. Legend is as in Figure 2.

therefore the corresponding transcript sequences in *X. tropicalis* are more difficult to annotate and were dropped out as "transcribed locus". A great deal of annotation is required to enrich the data available in both species.

7. SUMMARY AND PERSPECTIVES

In this review, the current knowledge of immune gene expression during metamorphosis was presented. As we have seen, our knowledge of the anatomy of the amphibian "immunome" is still fragmentary. Already, a wealth of data sits in cDNA and genome sequences but curated annotations are missing. Since most of the gene products involved in immune functions are rapidly evolving, making sense of these sequences is not easy and we should not expect too much from automatic annotations. A rapid formulation of hypothesis can be made starting from the current knowledge of mammalian immune system physiology and the recent surge of genomic data for both *X. laevis* and *X. tropicalis*. As a first example, I surveyed

available EST data with the aim to rapidly identify immune genes expressed during development and metamorphosis in *Xenopus*. EST surveys are compatible with microarray data, much alike any the kind of gene-oriented data such as functional classification and genome location. However, such a survey can not be directly compared to microarray experiments where, in most cases, one looks at the genes expressed in sample A but not B and vice-versa. Therefore mining EST data helps microarray analysis by providing independent information on the transcriptome.

As a first step, I focused on genes for which the EST evidences are abundant. A second step would be to progressively add cases of moderately or weakly expressed genes. If transcripts represented by two or more ESTs in *X. tropicalis* are selected, a dataset composed of more than 8,000 entries, mostly redundant, is produced. Thus, a significant amount of data integration is needed to build an exhaustive list of the immune genes in *Xenopus*. Such an undertaking is a component of the annotation of *X. tropicalis* the genomic sequence.

In parallel, we can use the annotation of human gene products made with the gene ontology thesaurus (44), selecting genes playing a role in the immune system process (GO:0002376), the immune response (GO:0006955) or the immune system development (GO:0002520) and identify *X. tropicalis* orthologs. Using the ENSEMBL BioMart tool (www.ensembl.org), such a query leads to the identification of 364 genes associated with immune responses out of 800 human genes annotated as such. What are the patterns of expression of these genes during metamorphosis is now a question that can be asked in experimental terms, either using EST counts as presented before, or more directly using transcriptomic tools (microarrays for example). The outcome of the EST survey presented here can be integrated to sequence similarity-oriented studies in order to better delineate the "immunome" of an amphibian. The regulatory network in which the immune genes are embedded can be probed using the *Xenopus* model to answer questions on the evolution of the immune system development.

An important topic concerns the interplay between immune functions and thyroid axis during amphibian metamorphosis. More research on the tadpole immune system are required if we want to understand the reasons of amphibian decline and develop alternative toxicology testing for endocrine disruptors.

8. ACKNOWLEDGEMENT

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Abbreviations: MHC: Major Histocompatibility Complex; cDNA: complementary DNA; EST: Expressed Sequence Tags; TH: Thyroid Hormone; TRH: Thyrotropin Releasing Hormone

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