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Ontogenetic onset of immune-relevant genes in the common sole (*Solea solea*)

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ABSTRACT

Fish are free-living organisms since initial stages of development and are exposed to numerous pathogens before their lymphoid organs have matured and adaptive immunity has developed. Susceptibility to diseases and juvenile mortality represent key critical factors for aquaculture. In this context, the characterization of the appearance kinetics of the immune system key members will be useful in understanding the ability of a particular species in generating immune protection against invading pathogens at different developmental stages.

The present study characterized, for the first time, the transcriptional onset of un-explored relevant genes of both innate and adaptive immune system during the *Solea solea* ontogenesis.

Gene expression profiles of immune relevant genes was investigated, by means of DNA microarray, in ten developmental stages, from hatching (1 day post-hatching, dph) to accomplishment of the juvenile form (33 dph).

The obtained results revealed that transcripts encoding relevant members of innate immune repertoire, such as lysozyme, AMPs (hepcidin, β-defensin), PPRs and complement components are generally characterized by high expression levels at first stages (i.e. hatch and first feeding) indicating protection from environmental pathogens even at early development.

Transcription of adaptive immune genes (i.e. Class I and class II MHC, TCRs) differs from that of the innate immune system. Their onset coincides with metamorphosis and larvae-to-juvenile transition, and likely overlaps with the appearance and maturation of the main lymphoid organs. Finally, data collected suggest that at the end of metamorphosis *S. solea* cell-mediated immune system hasn't still undergone full maturation.

Keywords: *Solea solea*, ontogeny, innate immunity, adaptive immunity, transcriptome, larval development, gene expression.

1. INTRODUCTION

Teleost represent the largest class of vertebrates with more than 40,000 species whose diversity allowed their adaptation to every aquatic environment. This heterogeneous group of organisms is the earliest vertebrate group that possess an immune system comparable to higher vertebrates, with an acquired immune system consisting of B- ant T-lymphocytes and memory formation (Fischer et al. 2005, Rauta et al. 2012). However, in contrast to mammals, fish are free-living organisms since initial stages of development and are exposed to numerous pathogens before their lymphoid organs have matured and adaptive immunity has developed.

Immunocompetence is a feature acquired by the organism during ontogeny. For survival, newly hatched fish rely on their innate immune repertoire (Rombout et al. 2005) that acts, in a non-specific manner, as the earliest mechanism that defends the host from infection. However, the development of long lasting immunological memory depends on the ability to activate the adaptive immune system (Øvergard et al. 2011).

A better understanding of the mechanisms related to innate immune responses is crucial for the development of effective approaches for disease management. In the same way, a proper knowledge of adaptive immune system is important since memory responses are possible targets for prevention strategies and it has been proven that immunostimulation before the immune system is fully developed induce tolerance resulting in the lack of response to later stimulation (Patel et al. 2009).

In recent years, considerable progress in the understanding of the development of the immune system in fish has been made. Extensive numbers of studies have been reported on the ontogeny of lymphomyeloid organs of marine fish and freshwater species (Patel et al. 2009, Cunha et al. 2008, Mulero et al. 2007, Falk-Petersen 2005 and references herein). In addition, studies on relevant genes of both innate and adaptive immunity have been conducted in many commercial fish species, such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), Japanese flounder (*Paralichthys olivaceus*), channel catfish (*Ictalurus punctatus*), turbot (*Scophthalmus maximus*), Atlantic halibut (*Hippoglossus hippoglossus*).

Although the literature in this field is getting extremely wide, few are the studies focused in analyzing, in a comprehensive way, the ontogeny of immune-relevant genes. Due to the importance of disease resistance in the fish industry, the characterization of the appearance kinetics of the immune system key members will be useful in understanding the ability of a particular species in generating immune protection against invading pathogens at different developmental stages.

The common sole (*Solea solea*) is a promising candidate for the European aquaculture, due to its high flesh quality, high market value and increasing demand by consumers (Parma et al. 2013). As for

other flatfish species, however, several critical bottlenecks remain to be solved in order to establish large scale sole farming production: susceptibility to diseases and juvenile mortality represent key critical factors for sole aquaculture.

Although increasing attention has been devoted on characterizing the transcriptome of this species (Ferraresso et al. 2013, Mazurais et al. 2014, Benzekri et al. 2014), still few information is available about its immune repertoire.

To overcome this limitation, the present study characterized and assessed the transcriptional onset of un-explored relevant genes of both innate and adaptive immune system during the *S. solea* ontogenesis, from hatching to accomplishment of the juvenile form. The obtained findings shed light on the development of common sole defense machinery and demonstrated that the main players of innate immunity are present during early developmental stages.

2. MATERIAL AND METHODS

2.1. S. solea trancriptome and in silico identification of immune related transcripts

Similarity analyses were performed on a total of 225,944 *S. solea* transcripts (22,252 isotigs/contigs and 203,692 singletons) obtained through Roche 454 GS FLX pyrosequencing, starting from a normalized cDNA library constructed by pooling larval stages (1, 4, 6, 8, 11, 13, 20, 33 dph) and adult tissues as described by Ferraresso et al. (2013). All sequences are stored in the public database Transcriptome Shotgun Assembly Sequence Database (TSA) under accession number GAAQ00000000.

The Basic Local Alignment Search Tool (BLAST) was used to perform annotation of *S. solea* isotigs/contigs and singletons (Ferraresso et al. 2013). Briefly, blastx analyses (cut off e-value of < 1.0 E-5) against NCBI amino acid nr database, SWISSPROT database and high quality draft protein databases of *Danio rerio, Gasterosteus aculeatus, Oryzias latipes, Fugu rubripes, Tetraodon nigroviridis, Homo sapiens,* and *Mus musculus,* available on Ensembl Genome Browser (release 56), were performed. In addition, dedicated searches through TBLASTN, using known teleost immune genes families as queries, were performed. Default algorithm parameters were modified using a less stringent e-value in order to score sequences with low homology. Identity of all contigs identified as potentially encoding immune-related genes were further screened for confirmation by FASTA searches (Pearson W 2004). *S. solea* sequences showing an e-value lower than 10 E-15 were manually checked and, whenever possible, prolonged at 5' and 3' ends by overlapping.

2.2. Solea solea larvae rearing and sampling

S. solea larvae employed in the present study are part of an experiment already described by Ferraresso et al. 2013. Briefly, all animals came from one batch of fertilized eggs obtained from spontaneous spawning of a broodstock maintained at the Laboratory of Aquaculture, Department of Medical Veterinary Sciences, University of Bologna, Italy.

Newly hatched larvae were maintained in a single incubator until mouth opening at 4 days post hatching (dph) and then allocated in three flat bottom 280-liter square tanks (2000 larvae tank−1). Animals were reared until 33 dph as described by Ferraresso et al. (2013).

Larvae were randomly collected at 1, 4, 6, 8, 11, 13, 15, 18, 24 and 33 dph. The onset of metamorphosis occurred at 13-14 dph (start of left eye migration) and ended at 24-25 dph (completion of left eye migration and visibility of left orbital arch on the dorsal side). Larvae were sacrificed by anesthetic overdose and sampled as described by Ferraresso et al. (2013). Euthanasia method and all experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with the European Community Council directive (86/609/ECC).

2.3. RNA extraction

Ten (10) developmental stages, as listed above, from hatching until completed metamorphosis, were used in the present work to characterize the larval transcriptome of *S. solea* by means of DNA microarray (as described later). Eight stages (i.e. 1, 4, 6, 11, 13, 18, 24, 33 dph) were previously employed (Ferraresso et al. 2013) while two (2) additional time points, 8 and 15 dph, were added in the present study. Total RNA was extracted from pools of *S. solea* larvae using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's specifications.

Each pool of larvae from 1 to 15 days post hatching (dph) contained approximately 10 individuals while from 18 to 33 dph pools were constituted by 5 larvae each. For each sample, RNA concentration was determined using a UV-Vis spectrophotometer NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, USA). RNA integrity and quality was then estimated on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and RNA integrity number (RIN) index was calculated for each sample, only RNAs with RIN number > 8.5 were further processed.

2.4. Solea solea oligonucleotide microarray

Gene expression analyses were performed using the Agilent-036353 *Solea solea* oligo microarray (GEO accession: GPL16124). This platform represents 15,385 unique transcripts, mainly represented by one probe (60 nt*) in situ* synthesized onto the array (Ferraresso et al. 2013), using Agilent non-contact ink-jet technology (4 x 18K format, including default positive and negative controls). A single dye (Cy3) labeling scheme was implemented, a mixture of 10 different viral poly-adenilated RNAs (Agilent Spike-In Mix) was also added to each RNA sample to monitor labeling and hybridization quality as well as microarray analysis work-flow. Sample labelling and hybridization were performed as reported in Ferraresso et al. (2013). Processed slides were scanned at 5 µm resolution using an Agilent G2565BA DNA microarray scanner. Default settings were modified to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The two linked images generated were analyzed together, data were extracted and background subtracted using the standard procedures contained in the Agilent Feature Extraction (FE) Software, version 9.5.1.

New microarray experiments were conducted on two developmental stages, 8 and 15 dph, by processing four (4) pools per stage. Newly produced microarray data were normalized together with the previous from Ferraresso et al. (2013) (i.e. 1, 4, 6, 11, 13, 18, 24 and 33 dph, 4 pools/stage). The normalization procedure was performed using R statistical software, microarray data were cyclic lowess normalized across all arrays. All raw and normalized gene expression data are available at the Gene Expression Omnibus (GEO) online microarray data repository under accession GSE81944.

2.5. Phylogenetic analyses

Phylogenetic trees were inferred using Bayesian inference (BI) and Maximum Likelihood (ML) methods (Felsenstein 2004). The BI tree was obtained with MrBayes 3.2.6 (Ronquist et al. 2012). Two simultaneous runs, each of four chains, were performed in each dataset analysis. Each run consisted of 2,000,000 generations, and trees were sampled every 200 generations (trees generated = 2 x 104). Stationarity was considered to be reached when the average standard deviation of split frequencies was less than 0.005. Burn-in was very stringent and only the last 2,000 generated trees were used to compute the majority-rule posterior consensus trees. The ML analyses were performed with Phyml 3.0 program (Guindon and Gascuel 2003), non-parametric bootstrap resampling (1000 replicates) was also performed to evaluate the robustness of tree topology.

For each amino acid alignment, the most appropriate model of protein evolution was selected using the Akaike information criterion as implemented in ProtTest v1.4 (Abascal et al. 2005).

3. RESULTS

In silico analysis of *S. solea* larval transcriptome led to the identification of 149 immune-related transcripts (listed in Supplementary file 1), many of which are represented by complete cDNA sequences that were identified for the first time in this species.

Herein we report on the characterization of those transcripts encoding some of the major proteins involved in both innate and adaptive immune system. Their transcriptional onset was investigated by means of DNA microarray in *S. solea* larvae from hatching to accomplishment of the juvenile form.

In order to facilitate the results discussion, these proteins have been subdivided into functional categories, as follows.

3.1. INNATE IMMUNE SYSTEM

3.1.1. Lytic enzymes

Lytic enzymes are important defence elements especially against bacteria (Magnadóttir, 2006), in the present study specific blast searches were conducted in order to identify transcripts coding different classes of lytic enzymes and their expression during larval development has been assessed.

In total, three transcripts coding C-type lysozyme (c-lyz) family members herein referred to as SsoC-lyz1(isotig00798), SsoC-lyz2 (isotig00800) and SsoC-lyz3 (isotig06704), and two related to G-type lysozyme (g-lyz) family members, SsoG-lyz1 (isotig19917) and SsoG-lyz2 (isotig13329), were identified in *S. solea*.

Gene expression during larval development of c-type (isotig00798 and isotig06704) and g-type (isotig19917 and isotig13329) *S. solea* lysozymes were also assessed by DNA microarray. Marked differences in expression profiles were observed between c-lyz and g-lyz members (see Figure 1). Both c-lyz transcripts showed a strong increase in expression levels over time, showing at 33 dph a fold-change of 121-fold and 638-fold for SsoC-lyz3 and SsoC-lyz1, respectively.

Slightly different expression profiles were appreciated for g-lyz members, SsoG-lyz1 showed a rapid increase of mRNA levels soon after start feeding (4-8 dph, 37-fold) followed by a decrease during and after metamorphosis, while SsoG-lyz2 is characterized by a gradual increase of expression until 13 dph (6.5-fold compared to 1 dph), a steady state during metamorphosis, and a decrease at 33 dph.

A second class of lytic enzymes are cathepsins (CTSs), a complex superfamily of lysosomal proteases that participate in many physiologic and pathophysiologic cellular processes. Among the different classes of CTSs, cathepsin L (CTSL) has a documented role in immune response in both vertebrate and invertebrate.

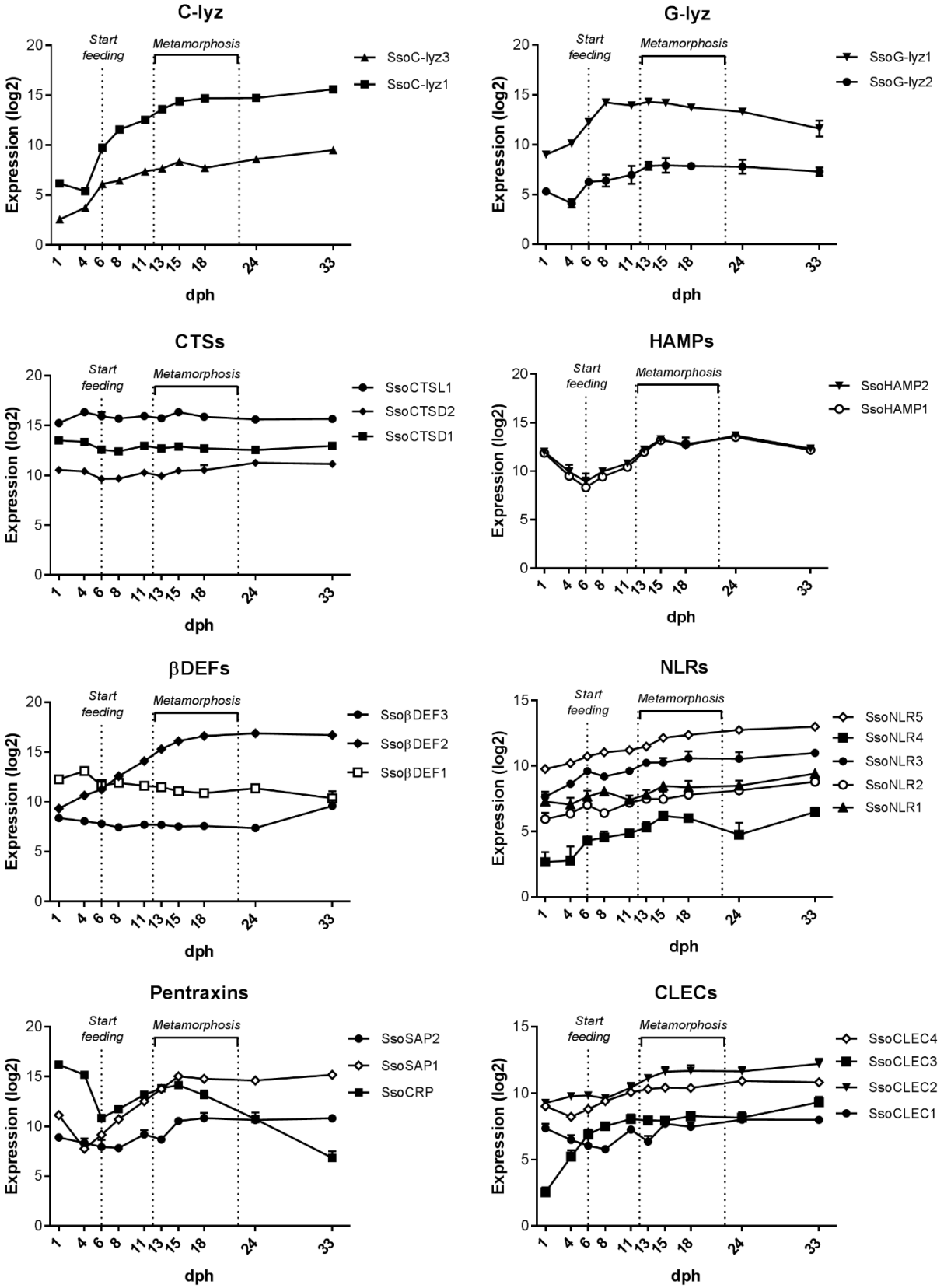


Figure 1. **Temporal expression of innate immune genes.** Gene expression levels (log2), from 1 to 33 dph, of *S. solea* transcripts coding innate immune genes. For each developmental stage, Standard deviation (SD) across biological replicates was calculated and represented as error bar, however in many points the error bar would be shorter than the height of the symbol and was not represented.

One cathepsin L gene was identified from the transcriptome of *S. solea* using *tblastn* searches, from now referred to as SsoCTSL1(isotig05294). SsoCTSL1 deduced protein sequence exhibited a conserved mature peptide with the catalytic triad of amino acids (Cys, His and Asn) usually present in its active site (see Supplementary file 2). Pairwise similarities between SsoCTSL1 and CTSL genes from other teleost species revealed 94% and 91% identity with *Cynoglossus semilaevis* (AEL31666) and *Epinephelus coioides* (AEN28617) CTSL, respectively. SsoCSTL1 gene expression pattern during early development revealed a high and constitutive expression from hatching to metamorphosis (see Figure 1).

Within cathepsin superfamily, also cathepsin D (CTSD) has a suggested, albeit still uncharacterized, role in immune response. In total two transcripts coding cathepsin D were identified in *S. solea*, namely SsoCTSD1 (isotig00536) and SsoCTSD2 (isotig07059). SsoCTSD1 and SsoCTD2 are not full length transcripts, since they encode 131 and 155 amino acids, respectively. However they both contain conserved Asp residue (D281) known to be involved in the CTSDs catalytic site (data not shown) and show 96.2% (SsoCTSD1) and 92.9% (SsoCTSD2) sequence identity with *Lates calcifer* CTSD (ABV59077) and *H. hippoglossus* CTSD (ABI85390), respectively. Gene expression analysis revealed a marked difference between SsoCTSD1 and SsoCTSD2 during larval development. SsoCTSD1 shows a pick of expression at 4 dph and then it maintains high and stable mRNA levels over time, while SsoCTSD2 shows a drop in expression prior to start feeding (1.9-fold at 6 dph compared to 1 dph) and then an increase (3-fold) during metamorphosis (see Figure 1).

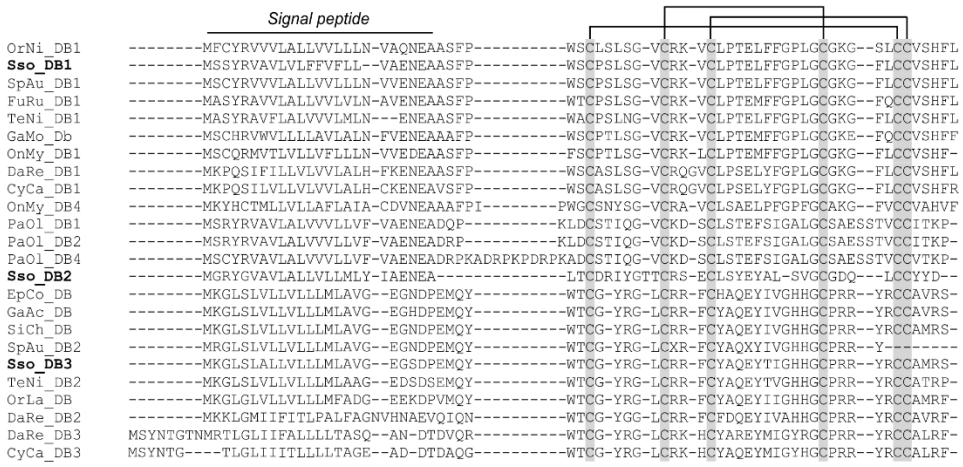
3.1.2. Antimicrobial peptides (AMPs)

Sequence analysis of *S. solea* contigs allowed the identification of several antimicrobial peptides (AMPs) belonging to different classes.

Two contigs encoding partially hepcidin antimicrobial peptides (hamp), namely SsoHAMP1 (isotig22007) and SsoHAMP2 (isotig03373), were identified showing significant match with *S. aurata* hepcidin 1 (ABV01929.1, 81% identity) and *S. maximus* hepcidin-2 (Q5CAJ5, 57.8% identity), respectively. Both transcripts show the same expression profile, mRNA levels are higher at hatching (1 dph) followed by a decrease at 4 and 6 dph and, after that period, transcription patterns show a marked increase prior to (15 dph, >20-fold compared to 6 dph) and during metamorphosis (24 dph, ~26-fold), and, finally, a new decline at 33 dph (see Figure 1).

It's worth of notice that *S. solea* contigs encode two not overlapping portions of hamp peptides and, even in light of their identical transcription profiles, it cannot be excluded they are sequences related to the same gene.

A.



B.

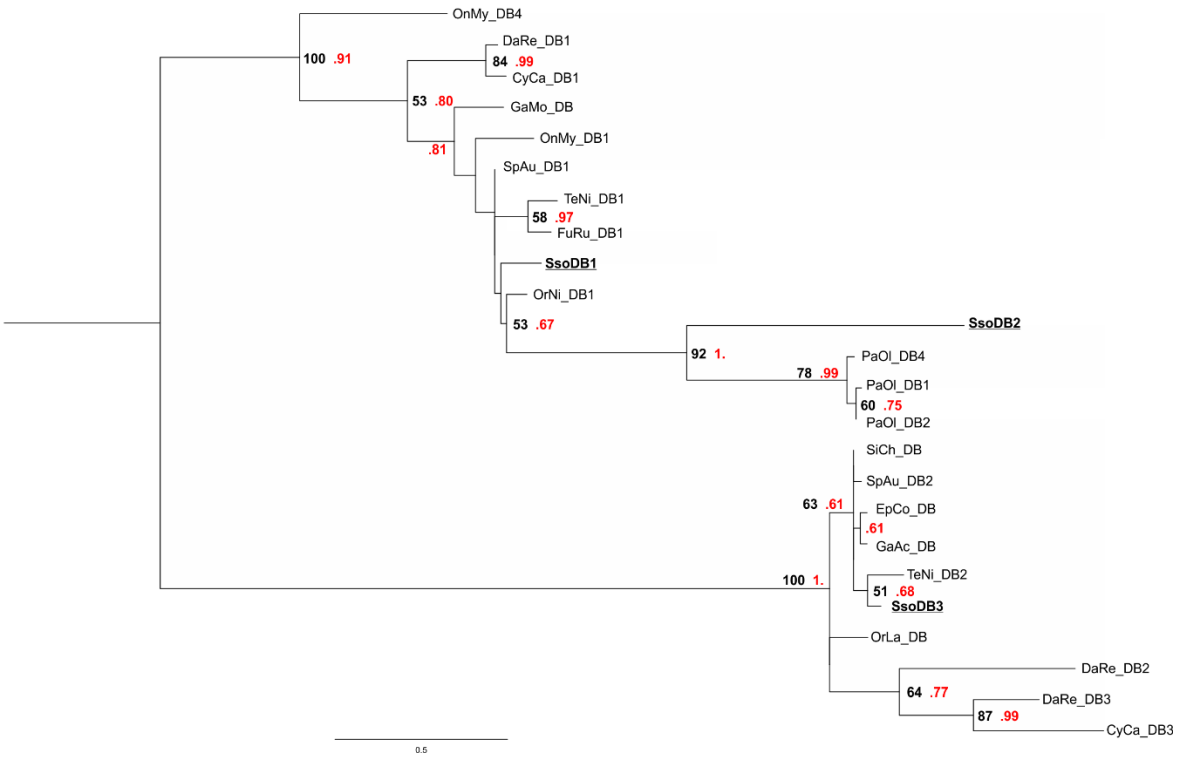


Figure 2. Phylogenetic analysis of *S. solea* β-defensin genes. A) Multiple alignment of the deduced amino acid sequences of *S. solea* (**Sso**) and other teleost β-defensin (DB) genes. Published DB sequences described from *Danio rerio* (**DaRe_DB1**, CAJ57442.1; **DaRe_DB2**, NP_001075023.1; **DaRe_DB3**, NP_001075024.1), *Oncorhynchus mykiss* (**OnMy_DB1**, CAK54950.1; **OnMy_DB4**, CAR82092.1), *Oryzias latipes* (**OrLa_DB**, ACG55699.1), *Paralichthys olivaceus* (**PaOl_DB1**, ADA84138.1; **PaOl_DB2**, ADA84139.1; **PaOl_DB4**, ADA84141.1), *Cyprinus carpio* (**CyCa_DB1**, AEZ00740.1; **CyCa_DB3**, AGZ03658.1), *Epinephelus coioides* (**EpCo_DB**, AAN02164.1), *Gasterosteus aculeatus* (**GaAc_DB**, ENSGACP00000027373), *Tetraodon nigroviridis* (**TeNi_DB1**, CAJ57644.1; **TeNi_DB2**, CAG00590.1), *Fugu rubripes* (**FuRu_DB1**, CAJ57646.1), *Gadus morhua* (**GaMo_DB**, AEB69787.1), *Oreochromis niloticus* (**OrNi_DB1**, AGW83444.1), *Siniperca chuatsi* (**SiCh_DB**, ACO88907.1), *Sparus aurata* (**SpAu**, unpublished) were included in the alignment. Cysteines (C) involved in disulphide bridge formation are highlighted with a connection showing which pairs form bonds. **B)** Phylogenetic tree showing the relationships between teleost β-defensin. Branch lengths correspond to the number of aminoacid substitutions, with the scale indicated at the bottom of the figure. Black numbers represent bootstrap values (values less than 50 are not shown) while red numbers refer to Bayesian Inference posterior probabilities. These latter values are provided in a compressed way (e.g. 1. for 1.00 ; .99 for 0.99).

Three full lenght transcripts encoding putative β-defensin were found, namely SsoβDEF1 (isotig03407), SsoβDEF2 (isotig19582) and SsoβDEF3 (isotig18247). They all share the common features of vertebrate defensins, including a small size with a precursor molecule ranging from 59 to 65 aa, six conserved cysteines in the mature region and the presence of a negatively charged glutamic acid residue in the middle of the mature peptide, a feature peculiar of the fish β-defensin (Figure 2A). MatGAT software was employed to determine the homology across *S. solea* β-defensin: the highest identity/similarity was appreciated between SsoβDEF1 and SsoβDEF2 (38.8% identity and 56.9% similarity), while lower identities/similarities were appreciated when comparing SsoβDEF3 with SsoβDEF1 and SsoβDEF2 (29.9%/47.7% and 26.2%/47.6%, respectively).

In order to analyze the evolutionary relationship of *S. solea* β-defensin, phylogenetic analyses were conducted (by applying the JTT+I+G evolutionary model) considering all publicly available teleost β-defensin proteins. Trees produced by ML and BI methods share the same topology (Figure 2B). As for other species, evolutionary analysis divided *S. solea* defensins into two major clusters; the first cluster groups SsoβDEF1 and SsoβDEF2, while SsoβDEF3 belongs to the second one. A strong relationship can be noticed between SsoβDEF2 and *P. olivaceus* β-defensins (bootstrap support >90%, posterior probability=1.0), the only other flatfish species represented in the dataset.

Transcriptional onset of all SsoβDEFs during ontogeny was investigated. *S. solea* β-defensin transcripts showed patterns of expression markedly different across developmental stages (Figure 1). SsoβDEF1 showed the highest expression levels soon after hatching (1-4 dph), followed by a decrease over time; SsoβDEF3 mRNA levels are generally low from 1 to 24 dph, followed by peak of expression (4.9-fold) at 33 dph, while SsoβDEF2 shows a considerable increase of expression over time (~187-fold between 1 and 24 dph).

3.1.3. Pattern Recognition Receptors (PPRs)

Nod-like receptors (NLRs) and Toll-like receptors (TLRs) are two major forms of innate immune sensors, which provide immediate responses against pathogenic invasion or tissue injury.

In silico analysis of *S. solea* transcriptome identified at least 5 different NLRs herein named SsoNLR1 (isotig00221), SsoNLR2 (isotig11055), SsoNLR3 (isotig18107), SsoNLR4 (isotig08270) and SsoNLR5 (isotig07671). Sequence analysis of SsoNLRs highlighted a high sequence conservation with *Larimichthys crocea* NLRC3 (KKF31313) with identity percentages ranging from 65 to 91% (78 to 99% similarity).

Analysis of SsoNLRs gene expression through DNA microarray highlighted very similar patterns of expression during larval development for all five transcripts showing a gradual increase of expression over time with fold-changes (1 dph *vs* 33 dph) ranging from 4- to 13-fold for SsoNLR1 and SsoNLR4, respectively (see Figure 1). Even if the trend of expression along time is similar for all SsoNLRs, the

same is not true for their mRNA levels since a difference up to 100-fold can be appreciated, with SsoNLR4 exhibiting the lowest level of expression and SsoNLR5 showing the highest.

All similarity analyses failed to identify TLRs in *S. solea* transcriptome. Specific tblastn searches by using all teleost TLRs as query found significant matches with several sequences coding leucine-rich repeats but no TIR domains were detected even if using only teleost TIRs as query. Neither MYD88 (Myeloid Differentiation factor 88) nor TICAM1 (Toll-Like Receptor Adaptor Molecule 1) genes, two key members of the TLR signaling pathway were found in *S. solea*, while other effectors such as TIRAP (TIR domain-containing adapter protein), IRAK4 (Interleukin-1 receptor-associated kinase 4) and PI3K (Phosphoinositine-3-kinase) were identified. Those genes didn't show big variation in expression during larval development except for IRAK4 that exhibited a gradual increase of mRNA levels over time reaching its peak of expression at 33 dph (FC 6-fold compared to 1 dph, data not shown).

It's worth noting that similarity analyses conducted in both *S. solea* isotigs and singletons identified 9 transcripts coding Novel-immune type receptors (NITRs), a family of natural killer receptors recently identified in teleost. These transcripts were all singletons thus only incomplete sequence information were available and no data upon their gene expression have been collected.

3.1.4. Agglutinins and precipitins

Important components of the innate immunity are agglutinins and pentraxins that play a pivotal role in opsonization of invading pathogens and activation of the complement cascade.

Two contigs (isotig14311 and isotig19418) were identified in *S. solea* transcriptome encoding one C-reactive protein (CRP). SsoCRP deduced protein sequence is 221 aa long and exhibits the Pentraxin family signature H-x-C-x-[ST]-W-x-[ST], in which the conserved cysteine is involved in an inter-chain disulfide bond (see Supplementary file 2). Pairwise similarities between SsoCRP and CRP proteins from other teleost species display a high level of sequence conservation with highest identity shared with *L. calcifer* CRP (73.5% identity, 89.2% similarity) and *Siniperca chuatsi* CRP (70.3% identity, 87.4% similarity).

SsoCRP pattern of expression during development shows highest transcript levels at 1 dph, a pronounced drop in expression before start feeding (~40-fold, 1 dph *vs* 6 dph), followed by an increase before and during metamorphosis, with a peak at 15 dph (9.7-fold compared to 6 dph), and a successive decrease of expression thereafter (see Figure 1).

Similarity searches identified also two transcripts coding *S. solea* Serum amyloid P-component (SAP), herein referred to as SsoSAP1 (isotig15744) and SsoSAP2 (isotig13411). As for SsoCRP, these transcripts exhibit the conserved Pentraxin family signature and show 58% (SsoSAP1) and 68% (SsoSAP2) sequence identity with *Perca flavescents* SAP (ADX97225) and *Anoplopoma fimbria*

SAP (ACQ58218), respectively. During larval development, both SsoSAPS exhibit similar expression patterns, with a decrease of expression soon after hatching followed by a great increase of expression until 15 dph (~150-fold for SsoSAP1) or 18 dph (7-fold for SsoSAP2).

C-type lectins (CLEC) functions as an endocytic receptors and are involved in antigen uptake at the site of infection. This gene family counts many members that share a common protein fold and signature. In *S. solea* transcriptome four transcripts coding CLEC were identified, namely SsoCLEC1 (isotig10440), SsoCLEC2 (isotig04630), SsoCLEC3 (isotig01907) and SsoCLEC4 (isotig05634). SsoCLECs deduced amino acid sequences share a conserved C-type lectin domain signature, despite pairwise alignment showed a high level of sequence variation across SsoCLECs (data not shown). Gene expression analysis revealed similar trends for SsoCLEC2, SsoCLEC3 and SsoCLEC4, that showed a gradual increase of mRNA levels over time with fold-changes (1 dph *vs* 33 doph) ranging from 3.5- to 109-fold for SsoCLEC4 and SsoCLEC3, respectively (see Figure 1). Different is the expression profile of SsoCLE1 that showed a 3-fold decrease of expression from 1 to 8 dph, followed by a gradual increase thereafter (4.7-fold, 33 dph vs 8 dph).

Member of the CLEC family is also the Macrophage mannose receptor 1(MRC1), a protein that acts as phagocytic receptor for bacteria, fungi and other pathogens and mediates the endocytosis of glycoproteins by macrophages. *In silico* analysis identified one transcript (isotig03928) coding *S. solea* MRC1 (SsoMRC1) that showed consistent sequence identity (63%) with *Oreochromis niloticus* MRC1 (XP_005476589). SsoMRC1 pattern of expression during larval development is similar to that of SsoCLE1 with a 2-fold decrease of mRNA levels until 8 dph, followed by an increase until 24 dph (data not shown).

3.1.5. Complement cascade

The complement is a major humoral system of innate immunity. Members of both classical, lectin and alternative pathways have been identified in several teleost species and almost all the homologues of the mammalian complement components have been identified in fish model species (i.e. zebrafish, trout) (Nakao et al. 2011). *In silico* analysis of *S. solea* sequences let to the identification of at least 14 members of the complement system, all listed in Table 1, that show a high level of conservation across teleost species.

Transcription profiles during development highlighted different patterns of expression across complement components (Figure 3). C3 component, key molecule of the alternative pathway, shows a gradual increase of expression over time with a fold increase up to 43-fold between 4 and 33 dph. Similar evidence was found also for B/C2 with both transcripts showing a strong increase in expression during and after metamorphosis, and a peak at 33 dph (56- and 58-fold compared to 1 dph), while all transcripts coding C4 and MASP-1 show no difference in expression during

development. Worth of notice is the very high level of expression of C1q-B transcript, while no data are available for the C1q-A isoform. Terminal complement components C5, C6, C7, C8 and C9 are all characterized by a similar expression profile with an increase of expression until the onset of metamorphosis and a second increase after metamorphosis completion (Figure 3).

Table 1: Complement component members identified in *S. solea*

Component	Putative isoform	*S. solea* Seq ID	% Identity (% Similarity) with other teleost species
C1q	**A**	Isotig00144	39.5% (72.2%) *Salmo salar*
	B	Isotig02657	41.3% (77.9%) *Danio rerio*
B/C2		Isotig04319	72% (84%) *Larimichthys crocea* ACS83542
		Isotig01531	77% (86%) *Larimichthys crocea* ACS83542
C3		Isotig00233	77% (88%) *Sparus aurata*
		Isotig04636	78% (87%) *Sparus aurata*
		Isotig01819	73% (80%) *Sparus aurata*
		Isotig21141	73% (90%) *Sparus aurata*
C4		Isotig15108	57% (75%) *Oreochromis niloticus* XP_005472530
		Isotig08486	61% (73%) *Oreochromis niloticus* XP_005472530
		Isotig05325	65% (83%) *Oreochromis niloticus* XP_005472530
C5		Isotig10341	67% (82%) *Fugu rubripes* XP_003974669
C6		Isotig18274	80% (86%) *Epinephelus bruneus* AEM37769
C7	**A**	Isotig01050	74% (83%) *Oreochromis niloticus* XP_003446192
	B	Isotig10343	73% (79%) *Oplegnatus fasciatus* AFZ93893
C8α		Isotig01719	77% (86%) *Oplegnatus fasciatus* AFZ93888
C8β	**A**	Isotig16508	86% (92%) *Pseudopleuronectes americanus* AAT01914
	B	Isotig03362	81% (91%) *Paralichthys olivaceus* Q9PVW7
C8γ		Isotig07766	76% (85%) *Oryzias latipes* AGE44247
C9		Isotig12351	80% (86%) *Oplegnatus fasciatus* AFU81223
		Isotig07088	76% (86%) *Oplegnatus fasciatus* AFU81223
MASP-1		isotig13433	82% (92%) *Oreochromis niloticus* XP_005462795
CD59		Isotig15198	75% (82%) *Oryzias latipes* XP_005457336
Complement factor H		isotig00434	64% (76%) *Perca flavescens*
		Isotig13606	66% (80%) *Perca flavescens*

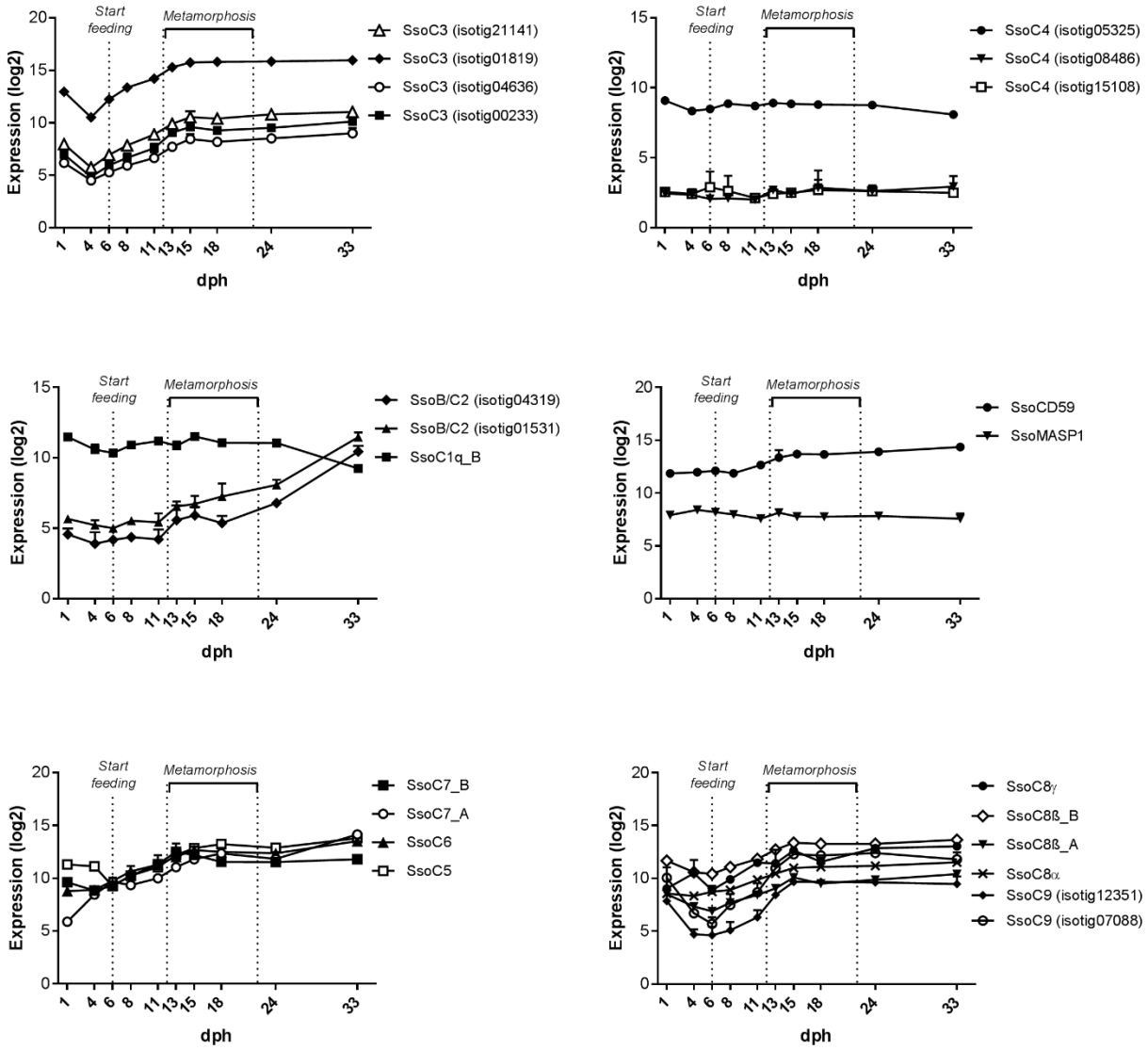


Figure 3. **Temporal expression of complement components.** Gene expression levels (log2), from 1 to 33 dph, of *S. solea* transcripts coding complement component members. For each developmental stage, Standard Deviation (SD) across biological replicates was calculated and represented as error bar, however in many points the error bar would be shorter than the height of the symbol and was not represented.

3.1.6. Cytokines cascade

Functional and genetic studies in many teleost species have proven that fish possess a network of signalling molecules, cytokines and chemokines, that function as important effectors of inflammatory responses. The *in silico* analysis of *S. solea* transcriptome identified a total of ten contigs encoding both CC-motif and CXC-motif chemokines showing different expression profiles during larval development (see Figure 4). All putative CC chemokines (isotig07900, isotig15888, isotig11434, isotig14436 and isotig06996) exhibit increasing expression levels over time with fold-changes up to 124-fold (33 *vs* 1 dph) while considerably different profiles were observed for CXC chemokines (isotig04373, isotig07783, isotig09845, isotig01371 and isotig10267).

When looking at inflammatory cytokines, similarity analyses identified one transcript (isotig14919) showing 75% identity with *C. semilaevis* Interleukin-1ß (IL1β; XP_008333362) and one contig (isotig10739) exhibiting 76% identity with *E. coioides* Transforming growth factor β1 (TGFβ1; ACV96791), from now referred as SsoIL1β, and SsoTGFβ1, respectively.

SsoIL1β gene expression is almost undetectable soon after hatching (1dph) and shows a dramatic increase of expression at the onset of metamorphosis (237-fold at 13dph), while SsoTGFβ1 maintains moderate and stable mRNA levels from 1 to 33 dph (see Figure 4).

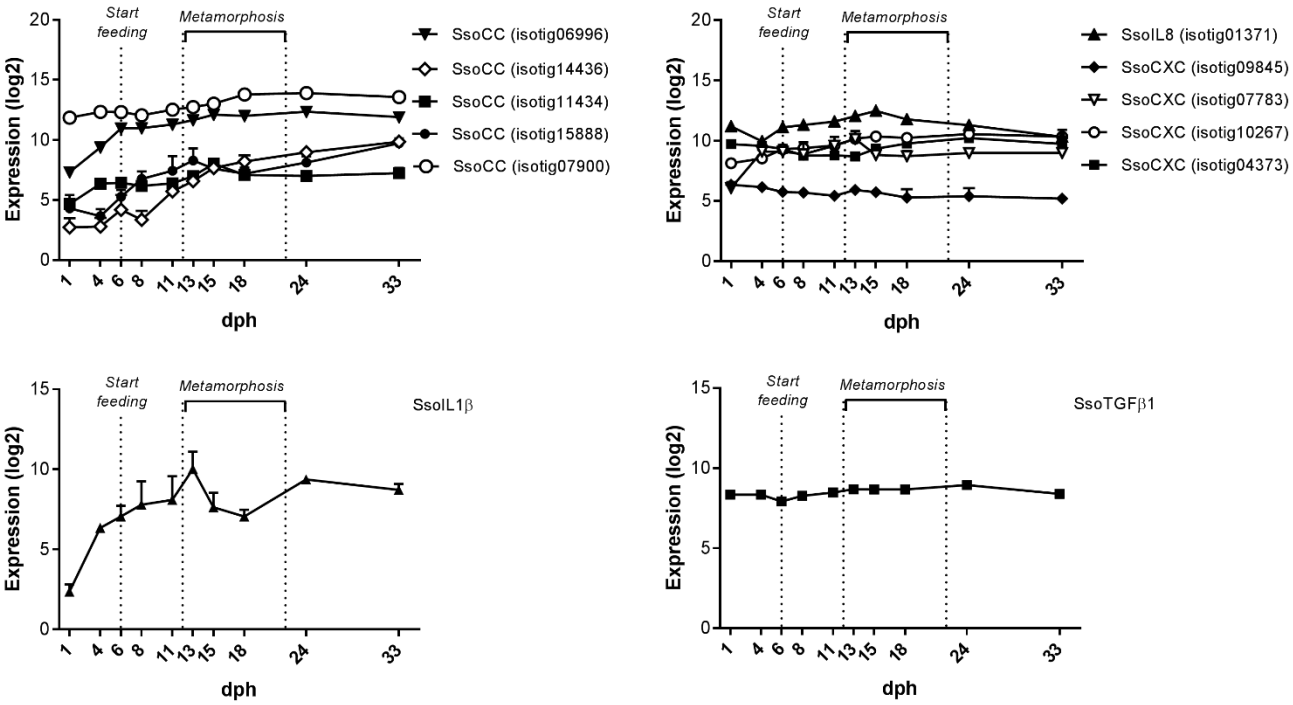


Figure 4. **Temporal expression of *S. solea* cytokines.** Gene expression levels (log2), from 1 to 33 dph, of *S. solea* transcripts coding cytokines. For each developmental stage, Standard Deviation (SD) across biological replicates was calculated and represented as error bar, however in many points the error bar would be shorter than the height of the symbol and was not represented.

3.2. ACQUIRED IMMUNE SYSTEM

3.2.1. Antigen presentation

Major histocompatibility complex (MHC) genes encode key molecules in the immune response and members of MHC complex can be distinguished in Class I or Class II MHC proteins.

Class I MHC is a non-covalently bound heterodimer composed of an MHC class Iα chain and β_2-microglobulin. MHC class Iα is expressed on all the nucleated cells and generally presents endogenous antigens to cytotoxic T lymphocytes (Cuesta et al. 2007). The MHC class I genes often exist as multiple copies, analysis of *S. solea* sequences lead to the identification of 3 different transcripts for MHC Iα, from now named SsoMHCIα_01 (isotig00026), SsoMHCIα_02 (isotig00049)

and SsoMHCIα_03 (isotig00066). Isotig00026 encodes the complete SsoMHCIα_01 protein sequence with leader peptide region, α1, α2 and α3 domains, connecting peptide, transmembrane domain and cytoplasmic domain (See Supplementary file 3), while isotig00049 and isotig00066 are not full-length transcripts and they encode putative proteins lacking the leader peptide and a portion of the α1 domain.

The three deduced MHC Iα protein sequences were aligned to other vertebrate MHC class I alpha proteins and showed all the features conserved across vertebrates, such as the presence of cysteines involved in the α2 and α3 intra-domain disulphide bridge (C125, C189, C225 and C285), a potential *N*-glycosylation site (NQT, position 111–113 in alignment) and conserved residues important for peptide and β2-microglobulin interaction (See Supplementary file 3).

Phylogenetic analyses were conducted (by applying the JTT+I+G evolutionary model) to determine the evolutionary relationship of sole MHC Iα genes (Supplementary file 3), and all *S. solea* alleles cluster together with bootstrap support and posterior probability and are in close relationship with the Japanese flounder and the turbot sequences, the other two teleost species belonging to the order Pleuronectiformes.

Class II MHC is a heterodimer composed of α and β chains, expressed in the surface of only a few cell types and generally presents exogenous antigens to T helper lymphocytes. *In silico* analysis of *S. solea* transcriptome identified two and three different isoforms for MHC IIα and MHC IIβ, respectively. SsoMHCIIα_01 (isotig01270) and SsoMHCIIα_02 (isotig01271) encoded full-length transcripts and both present Leader peptide, α1 and α2 domains, connecting peptide, transmembrane region and cytoplasmic domain (see Supplementary file 3).

Three different amino acid sequences were identified for *S. solea* MHC IIβ, namely SsoMHCIIβ_01 (isotig00164), SsoMHCIIβ_02 (isotig00165) and SsoMHCIIβ_03 (isotig00169), sharing 91-94% amino acid identity. SsoMHCIIβ_01 and SsoMHCIIβ_02 encoded full-length transcripts and possess Leader peptide, β1 and β2 domains, connecting peptide, transmembrane region and cytoplasmic domain, while SoSoMHCIIβ_03 sequence lack the Leader peptide.

The deduced *S. solea* MHC IIα protein sequences share 48.9-78.6% amino acid identity with other teleost species and similar results were obtained from the alignment of the deduced amino acid sequence of the sole MHC IIβ (46.8-73.6%). To evaluate the evolutionary relationships of the common sole MHC IIα and MHC IIβ, two phylogenetic trees (by applying the WAG+G+F evolutionary model) were conducted based on the protein sequences of teleost MHC II (Supplementary file 3). Both phylogenetic trees evidenced a close relationship with teleost belonging to the order Pleuronectiformes and *S. solea* MHCs grouped in a unique cluster with high bootstrap support and posterior probability.

Gene expression levels of both class I and class II MHC (each represented onto the microarray platform by one member) have been assessed from hatching to the end of the metamorphosis. MHC Iα, MHC IIα and MHC IIβ showed totally comparable patterns of expression, with mRNA levels barely detectable soon after hatching followed by a gradual increase during and after metamorphosis and a peak of expression at 33 dph, with fold-changes ranging from 7.5 to 106-fold (see Figure 5).

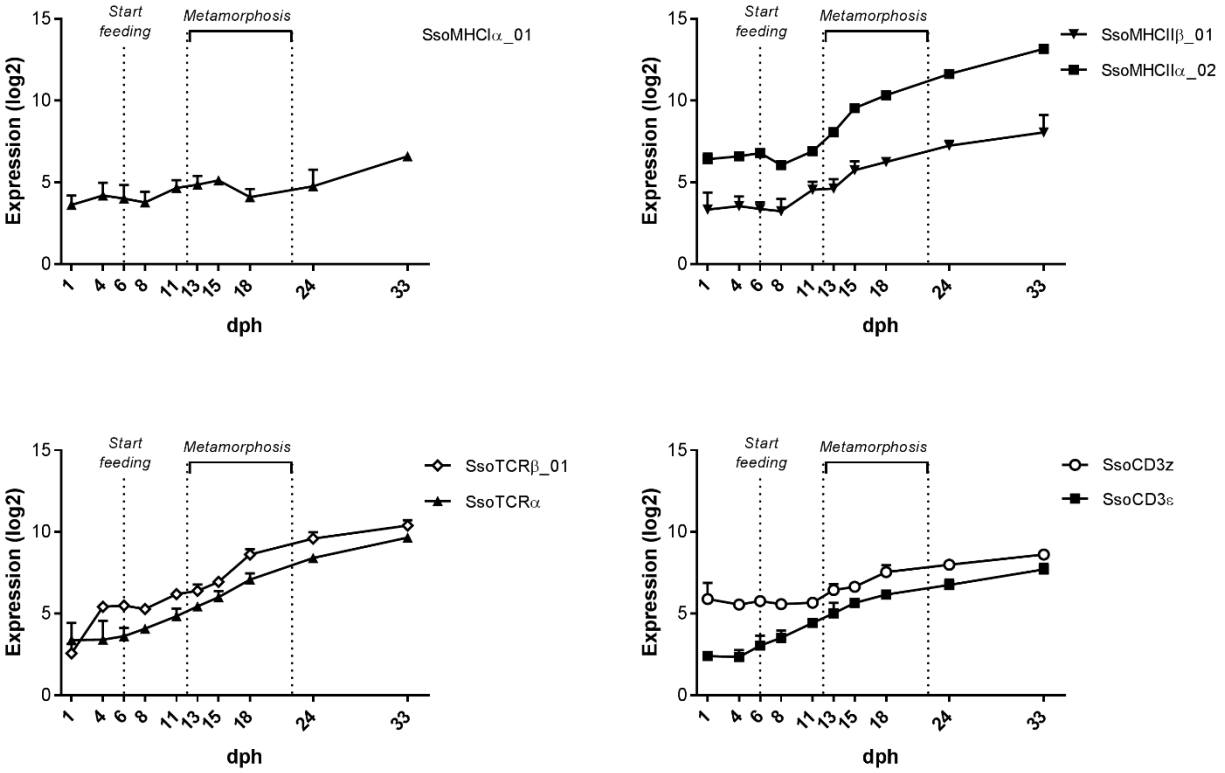


Figure 5. **Temporal expression of *S. solea* adaptive immune genes.** Gene expression levels (log2), from 1 to 33 dph, of *S. solea* transcripts coding adaptive immune genes. For each developmental stage, Standard Deviation (SD) across biological replicates was calculated and represented as error bar, however in many points the error bar would be shorter than the height of the symbol and was not represented.

3.2.2. Antigen recognition

Antigen-specific T-cell responses are initiated through the interaction of a T cell antigen receptor (TCR) and the corresponding peptide-MHC protein complex expressed on cell surface. TCRα and TCRβ genes have been characterized in both teleost and cartilaginous fishes (Castro et al. 2011 for a review). In the present study, one transcript (Isotig02715) was identified coding TCRα Constant region (Cα) and it exhibits 62% identity at the protein level with the orthologous sequence in *H. hippoglossus* (ACY54771.1). *S. solea* Cα possess several conserved structurally important features, such as a Cys residue in the CP domain, thought to form an inter-chain disulphide bond with the TCRβ chain in mammals, and the positively charged residues arginine (Arg) and lysine (Lys) in the

TM region, involved in the assembly and the surface expression of the TCR-CD3 complex (see Figure 6A).

Similarity analyses of *S. solea* transcriptome identified also two different TCRβ chains, SsoTCRβ_01 and SsoTCRβ_02 (Isotig00319 and Isotig00320, respectively). The two cDNAs, that codify the TCR Constant region (Cβ), share 81% identity at the protein level and are closely related to *H. hippoglossus* TCRβ (ACY54772.1, 63-61% aa identity). *S. solea* TCRβ Ig domains possess the conserved aminoacid motif S-R-L-R, which is required to form the TCR αβ heterodimer, and the phenylalanine (Phe_{250}) residue, which is considered important for TCR recognition (see Figure 6B).

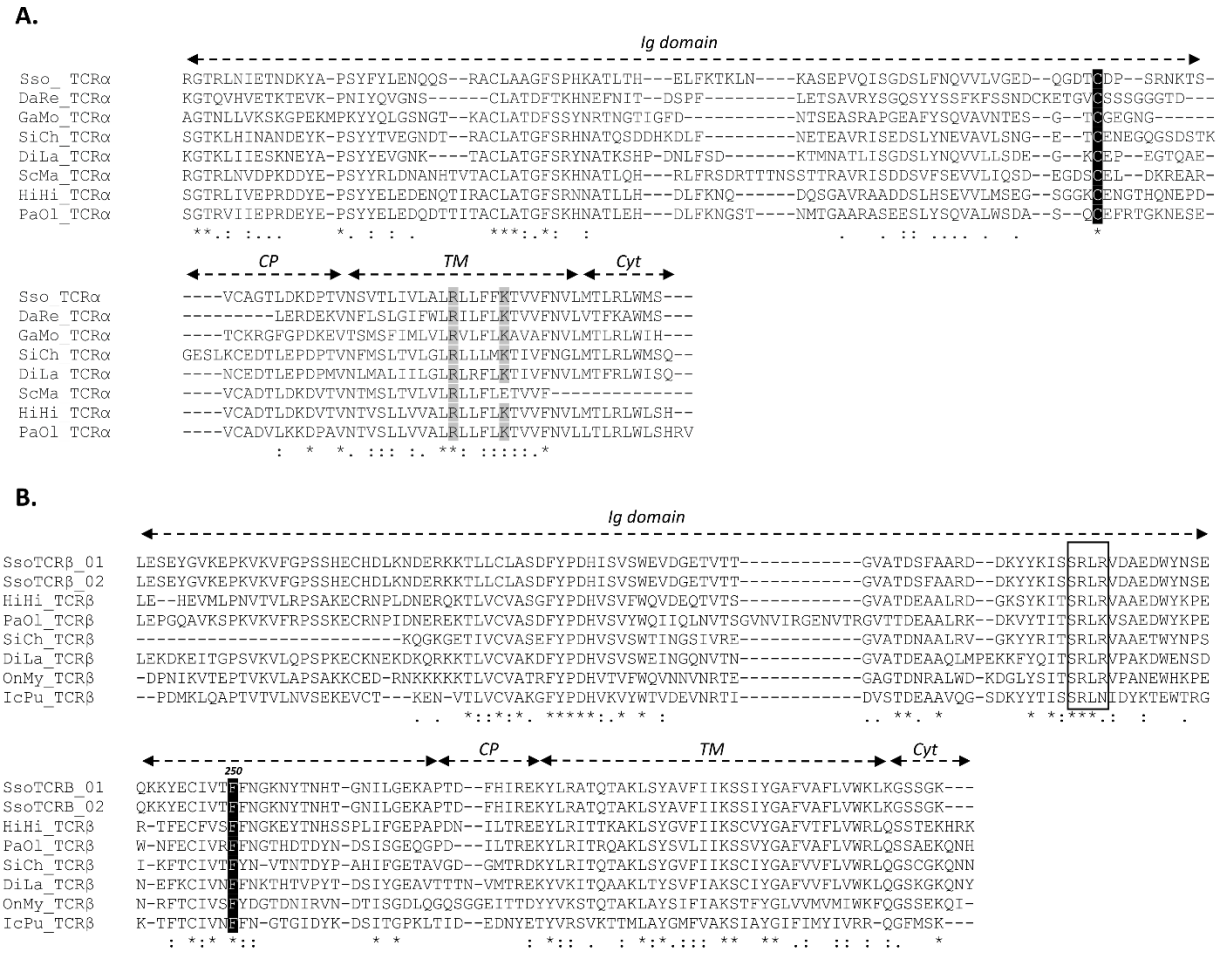


Figure 6. Multiple alignment of teleost TCR constant regions. A) Alignment of the deduced amino acid sequences of *S. solea* (**Sso**) and other teleost TCR Cα regions. Published TCRα sequences described from *Danio rerio* (**DaRe**, AAG31714.1), *Paralichthys olivaceus* (**PaOl**, BAB82531.1), *Gadus morhua* (**GaMo**, CAD28810.1), *Siniperca chuatsi* (**SiCh**, ABV60622.1), *Dicentrarchus labrax* (**DiLa**, AAV88084.1), *Scophthalmus maximus* (**ScMa**, AAP76193.1), *Hippoglossus hippoglossus* (**HiHi**, ACY54771.1) were included in the alignment. Highlighted in grey are residues involved in the assembly of the TCR-CD3 complex while conserved Cys residue is black shadowed. **B)** Alignment of the deduced amino acid sequences of *S. solea* (**Sso**) and other teleost TCR Cβ regions. Published TCRβ sequences described from *Hippoglossus hippoglossus* (**Hihi**, ACY54772.1), *Paralichthys olivaceus* (**PaOl**, BAB82607.1), *Siniperca chuatsi* (**SiCh**, ABV60623.1), *Dicentrarchus labrax* (**DiLa**, CBK52290.1), *Oncorhynchus mykiss* (**OnMy**, AAA92558.1), *Ictalurus punctatus* (**IcPu**, AAL07367.1) were included in the alignment. The core motif S-R-L-R is boxed while conserved Phe residue is highlighted in black. In both alignments, sequences are defined by the four-letter species abbreviation. At the bottom of each alignment, identical residues are indicated by " * ", conservative and less conservative residues are indicated by " : " and " . ", respectively.

T cell signaling complex in fish seems to include counterparts of mammalian CD3ε and CD3Z (mainly known as CD247). This complex plays an important role in coupling antigen recognition to several intracellular signal-transduction pathways. In the present study two full-length transcripts, one (isotig06954) coding SsoCD3ε and one (isotig14459) coding SsoCD3Z, were identified showing 52% identity at protein level with *H. hippoglossus* CD3ε (ACY54760.1) and 68% identity with *L. crocea* CD3Z (XP_010753352), respectively.

Changes in expression of *S. solea* TCRα, TCRβ CD3ε, and CD3Z during development were also assessed (see Figure 5), and all transcripts were proven to exhibit the same trend, with mRNA levels almost undetectable soon after hatching followed by a gradual increase over time with fold-changes at 33 dph ranging from 5.4- (CD3Z) to 217.8-fold (TCRβ).

4. DISCUSSION

Since the early stages, fish life occurs in the open environment. In particular, during the first critical transitions of larval development (i.e. hatching, mouth opening, first feeding) interactions between the developing teleost immune system and the environment intensify, increasing dramatically the need for efficient defence mechanisms against pathogens.

In the last decade, extensive numbers of studies have been conducted in order to improve the understanding of teleost immune system. Transcriptome sequencing through NGS strategies allowed a rapid increase of sequence information also in non-model species (Huang et al. 2015, Ali et al. 2014, Tong et al. 2015, Xia et al. 2010), however studies have been mainly limited to identify immune relevant genes and/or characterize their response to pathogen invasions, while little is known about their temporal appearance during development as a whole.

To date, information on the ontogeny of the teleost immune system is largely restricted to a few fish species and usually to a limited number of genes (Cecchini et al. 2013, Seppola et al. 2009, Rise et al. 2012, Nayak et al. 2011, Picchietti et al. 2009, Broekman et al. 2011), making it difficult a comparison between different species.

Herein, similarity searches led to the identification of 149 immune-related isotigs/contigs in a total of 225,944 *S. solea* sequences. Many of these transcripts are represented by complete cDNA sequences that were identified for the first time in this species. The identification of such a repertoire in common sole provided the basis for studying the transcriptional profiles of many immune components across ten (10) larval stages, from hatching to accomplishment of the juvenile form, and to address if genes that have an important role in immune defense are present early during development.

Innate immune elements

During evolution, marine eukaryotes have developed a wide array of anti-infective molecules and strategies by which they protect themselves against prokaryotic and viral attack (Smith et al. 2010). Innate immune system is the earliest immune mechanism that acts as first line of defense against all pathogens, and protect the host from invasion by other organisms in a nonspecific manner. It is known that teleost immunological capacity is limited in early embryos. The immune system is not completely developed during embryonic and larval stages, thus conferring to innate mechanisms a key role for survival.

Here, the temporal appearance during larval development of relevant members of innate immune repertoire, such as lysozyme, AMPs, PPRs and complement has been assessed.

Five lysozyme transcripts including three c-type and two g-type lysozymes have been identified in *S. solea*. Only a few studies assessed the ontogeny of lyz genes in teleost fish (Seppola et al. 2009, Nayak et al. 2011) reporting, in particular for g-type lysozyme, its transcription from embryos

onwards. A confirmation was found in the present study with SsoLYZs mRNA levels detectable from hatching and, with gene-specific trends, generally increasing after the start of the exogenous feeding. AMPs are host produced low molecular weight peptides (or small proteins), typically exerting a potent broad-spectrum activity against pathogenic bacteria, fungi, parasites, and viruses (Cole et al. 2000, Dezfuli 2010). Extensively studied in mammals, amphibians and invertebrates, these molecules have recently received attention in teleost, leading to the identification of many different families of AMPs in the last two decades (Smith et al. 2010 for a review). In the present study two classes of AMPs, hepcidin and β-defensin, have been identified in *S. solea*.

Hepcidin (HAMP) is a small cysteine-rich protein with not only antimicrobial activity but also pivotal function in iron homeostasis (Wang et al. 2012). It is known that HAMP genes can be distinguished into two isoforms with specific activities (Neves et al. 2015, Hilton et al. 2008). Due to the incomplete sequence information, no phylogenetic analyses were conducted on SsoHAMP transcripts, preventing us from defining whether this/these peptide/s belong to hamp1- or hamp2-type isoforms, the latter exerting primarily an antimicrobial function.

SsoHAMPs gene expression patterns during larval development show higher expression levels at hatching and a new increase in expression from 8 dph. This totally reflect what already observed in the Atlantic cod (i.e. *Gadus morhua*, Seppola et al. 2009) and in the half-smooth tongue sole (i.e. *C. semilaevis*, Wang et al. 2012), while is markedly different to what reported for the Medium Carp homologue (i.e. *Puntius sarana*, Das et al. 2015), where a stable expression from 6 h post fertilization onwards was observed. This might be due to isoform-specific differences in expression profiles, already described in teleost healthy and challenged tissues (Neves et al. 2015), as well as during ontogenesis (Martin-Antonio et al. 2009). In both Atlantic cod (Seppola et al. 2009), half-smooth tongue sole (Wang et al. 2012) and redbanded seabream (Martin-Antonio et al. 2009), some HAMP isoforms are expressed in embryos and at hatching followed by a decrease in expression in later stages, leading to the hypothesis that those genes might play a role in embryogenesis and early larval development, likely through their known function in iron homeostasis (Martin-Antonio et al. 2009, Shi et al. 2006).

Under this scenario, even for *S. solea* HAMPs, a non immunological function might explain their higher expression at hatching compared to immediately following stages, while the second increase of transcript levels is ascribable to the growing needs for antimicrobial defense at the commencement of exogenous feeding.

Beta-defensins are antimicrobial peptides that have an important role in innate immune responses at epithelial barriers. Herein, three full-length transcripts coding β-defensin have been identified, and like their counterpart in higher vertebrates and other teleost species (Zou et al. 2007, Casadei et al.

2009, Nam et al. 2010), they fall into two subgroups based on phylogenetic analysis. Noteworthy, *in silico* analysis of SsoβDEFs protein sequences highlighted that SsoβDEF2 possesses an anionic net charge.

In general, defensins are known as cysteine-rich cationic antimicrobial peptides. However, non-cationic defensins have been found in insects (Lai et al. 2004) and, recently, in *P. olivaceus* (Nam et al. 2010), where they have been reported to exert antimicrobial activity against Gram-positive bacteria, even though their mechanism of action is still unknown. In the present study, phylogenetic analysis reported SsoβDEF2 as closely related to non-cationic *P. olivaceus* β-defensins, but further investigations are required to verify if these sequence features contribute to their antimicrobial activities.

β-defensin gene expression in teleost has been assessed in tissues from healthy individuals (Zou et al. 2007) and/or following bacterial challenge (Casadei et al. 2009) reporting that, in the same species and under the same conditions, members of β-defensin family show different tissue distribution and patterns of expression (Zuo et al. 2007, Casadei et al. 2009). The same evidence was observed in the present study with SsoβDEF transcripts showing markedly different patterns of expression during development, suggesting a distinct role of these peptides in host defense.

Although different trends of expression were observed, all SsoβDEF transcripts show high transcription levels across all larval stages. β-defensin constitutive expression during early developmental stages was already reported in another teleost species (*i.e. P. olivaceus*, Nam et al. 2010), in insects (Wen et al. 2009) and higher vertebrates (Meyerholz et al. 2004, Meada et al. 2009). In human, β-defensins have also been proven *in vitro* to participate in keratinocytes growth/differentiation and in maturation of skin DCs (Frye et al. 2001, Ferris et al. 2013). In this context, expression patterns observed herein might suggest that these peptides, in addition to a primary function in protecting newly hatched fish from pathogenic assault, might play a role even in cellular growth/differentiation in developing fish larvae.

Vertebrates have evolved a vast array of both extracellular and intracellular pathogen recognition receptors (PRRs) for detecting and responding to pathogen-associated molecular patterns (PAMPs) (Hansen et al. 2011), and their role is critical to the initiation of both innate and adaptive immune responses.

Nod-like receptors (NLRs) constitute a multigene family of cytoplasmic PRRs in which three subfamilies can be distinguished; i) NLR-A, putative orthologous of mammalian NODs; ii) NLR-B, putative orthologous of mammalian NALPs, and iii) NLR-C that appears to be unique of teleost fish and is currently still not well defined (Zhu et al. 2013, Laing et al. 2008).

Herein, five different *S. solea* NLRs were identified, all showing consistent similarities to teleost NLR-C family members (data not shown). In human, Nod-like receptors have been proposed playing a crucial role not only in innate immune responses, but also in apoptosis and early development (Kim et al. 2016). All SsoNLRs are expressed early during development and their expression levels constantly increase over time. To our knowledge this is the first time that NLRs ontogeny is investigated in both teleost and higher vertebrates, and the present findings suggest that these receptors might take part in immune repertoire maturation and larval development.

Within PRRs, Toll-like receptors play a pivotal role in early recognition of pathogens as well as in the initiation of a robust and specific adaptive immune response. At least 10 TLRs have been identified in fish (Palti 2011, Pietretti et al. 2014) from several different orders including Pleuronectiformes (Yu et al. 2009, Hwang et al. 2010), and have been proven to be transcriptionally active even during embryogenesis in both teleost and higher vertebrates (Yu et al. 2009, Peterson et al. 2005, Kannaki et al. 2015).

Despite targeted Blast searches were carried out using entire TLR and TIR domain sequences from several teleost species, in the present study no putative TLR transcripts have been detected in *S. solea* larval transcriptome. Nevertheless some effectors molecules, such as TIRAP, IRAK4 and PI3K, that have an essential role in the TLR signaling cascade (Kawagoe et al. 2007), were found. Even though IRAK4 and PI3K are involved also in other biological processes such as IL1 signaling pathway (Suzuki et al. 2002, Sizemore et al. 1999), TIRAP seems to be specifically involved in signaling through the members of the TLR family and it could provide an indirect evidence of the transcriptional activation of TLR repertoire in common sole even if we could not detect any related sequence in our search.

A possible explanation of the lack of TLRs transcripts in our data could be given by the low sequencing depth of the obtained sole transcriptome. At the time of *S. solea* transcriptome sequencing (2011) the Roche 454 GS FLX technology was chosen mainly because of the average read length (300-600 nt), significantly longer compared to other platforms (Illumina and Solid) and more suitable for *de novo* sequencing/assembly. However, 454 technology produces 0.4-0.6 Gb per run that can be converted into an extremely low number of reads/run, explaining the difficulty of detecting/sequencing low represented transcripts, even if normalized cDNA libraries have been employed as template. If ever formulated, the hypothesis of the absence of TLR repertoire in *S. solea* was definitely ruled out by the study from Benzekri et al (2014) in which at least 7 TLRs were identified.

In recent years, a new multigene family encoding non-rearranging receptors belonging to immunoglobulin superfamily, called Novel immune-type receptors (NITRs), has been discovered and

their occurrence seems to be restricted to teleost species (Ferraresso et al. 2009, Yoder et al. 2010, Meng et al. 2014). Herein, NITR transcripts have been identified in *S. solea* larvae, unfortunately they were represented by only partial sequences and, so far, no further analyses have been conducted. However, this represents the first evidence of the presence of multiple NITR genes on Soleidae family. Recognition of potential pathogens can be mediated also by some humoral lectins, such as pentraxins, that function as effector factors by promoting their phagocytosis and activating the complement system. In vertebrates, pentraxin family is generally composed by two members, C-reactive protein (CRP) and Serum amyloid P (SAP).

CRP and SAP share extensive sequence homology, indicating that they evolved from a common ancestor and often, in teleost, these proteins are generally named as "pentraxin" or "pentraxin-like". However, CRPs and SAPs vary considerably in terms of ligand-binding specificity and in some species they were characterized based on their substrate selectivity (Huong Ciang et al. 2010). In the present study, three transcripts coding pentraxin family members were identified, having best match with either teleost CRP or SAP. To properly classify *S. solea* pentraxins, a phylogenetic analysis was conducted by using sequences of both CRP and SAP from several mammals and teleost. Phylogenetic tree showed that all pentraxin sequences grouped in two clusters, corresponding to mammalian SAPs and CRPs, with high bootstrap support, providing high confidence in the *S. solea* gene identity as initially revealed by BLAST searches (data not shown). To our knowledge, this is the first time that the transcriptional onset of both CRP and SAP transcripts have been investigated in teleost during larval development. The only available information is by Seppola and colleagues (2009), that investigated the gene expression profile of a pentraxin transcript during cod development. This study reported expression profiles comparable to *S. solea* homologues, with increased mRNA levels during embryo development, a drop in expression soon after hatching and a second increase during metamorphosis. The meaning of this trend of expression is unclear. In mammals, it has been reported that some pentraxin members are involved also in synapse formation and remodeling (Boulanger 2009). During metamorphosis, flatfishes dramatically transform from a symmetrical to an asymmetrical body shape, accompanied by eye migration, cranium deformation and tissue remodeling. In this context it is not unlikely that *S. solea* pentraxin play a role in neurological processes that take place during both early embryogenesis and sole metamorphosis.

The complement system modulates several physiological processes, from inflammation and pathogen opsonization to hematopoiesis, tissue regeneration and lipid metabolism (Forn-Cuni et al. 2014). In particular, it plays a central role in early pathogen defense and bridges the innate with the adaptive immune response (Sunyer, et al. 2003). There are three distinct pathways through which complement can be activated on pathogen surfaces; i) the Classical pathway, ii) the Alternative pathway and the

ii) Mannan-binding (MB)-lectin pathway (Janeway et al. 2001). These pathways depend on different molecules for their initiation, but they converge to generate the same set of effector molecules (terminal complement components). Multiple forms of complement component genes have been previously identified in fish, suggesting a key role for this system in aquatic organisms (Forn-Cuni et al. 2014).

In the present study, the main components of both classical, alternative and MB-lectin pathways have been identified, clearly indicating that all three pathways of the complement cascade are represented in the common sole. As expected, almost all members of the complement system, terminal components included, are expressed early during development and show an increase in expression over time, in particular after the start of the exogenous feeding, in a way totally compatible with the growing needs for efficient defense mechanisms against pathogens that accompany larval development. The only exception is represented by C1q, C4 and MASP-1 transcripts, main components of the classical and MB-lectin pathways, that exhibit no difference in expression, however they have a high/moderate constitutive expression in all larval stages analyzed, proving evidence that these pathways are already "active" during early development.

The third complement component (C3) is a central protein in all three overlapping pathways of complement activation (Wang et al. 2015). Herein, we report the identification of four different complement C3 transcripts in the common sole transcriptome. As already observed in other teleost species (Forn-Cuni et al 2014, Lange et al. 2004, Lovoll et al. 2007), gene expression analysis of SsoC3 genes during ontogeny showed for all transcripts similar patterns of expression with mRNA levels increasing soon after mouth opening (4 dph). Of particular interest, in both *S. solea* and zebrafish (Forn-Cuni et al 2014), a decreased expression of C3 transcripts soon after hatching can be appreciated, suggesting for this molecule a non-immunological function during embryo development as already demonstrated in mammalian species (Carmona-Fontaine et al. 2011, Jeanes et al. 2015).

Cytokines are a broad group of small proteins that act as intercellular mediators to regulate the immune response and are known acting as a bridge between innate and adaptive response. Release of pro-inflammatory cytokines is essential for the activation of an effective innate host defense, and subsequently for the modulation of adaptive immune responses. Among these, interleukin-1ß is one of the most studied pro-inflammatory cytokine, mainly produced by activated macrophages and blood monocytes (Lu et al. 2008). Once secreted, it initiates and enhances the inflammatory response by inducing the expression of pro-inflammatory molecules and adhesion molecules in diverse stromal/inflammatory cells (Apte et al. 2006). In addition to its function in inflammation, IL1β plays a pivotal role in the proliferation and differentiation of cells of both innate and adaptive immunity (Lu et al. 2008). Ontogenesis of IL1β was recently investigated in zebrafish by Dios et al. (2010) that

reported its transcriptional onset at 8 dpf. This is in agreement with our findings (mRNA levels barely detectable at hatching and increasing thereafter) and it is consistent with the primary role of IL1β in maturation of the immune system.

Contrary to IL1β, in the present study other crucial pro-inflammatory cytokines such as TNFα and IFNs were not identified. This can be linked to the biological function of these molecules. IFNγ is known to be involved in both innate and adaptive immunity (Robertsen 2006), nevertheless it is mainly secreted by activated NK cells and T-helper lymphocytes, thus it is likely that in both developmental stages and (not inflammatory) conditions employed for cDNA library construction this transcript was expressed at low levels and therefore not detectable. We could argue the same for TNFα, produced by a wide array of cells but, usually, mainly in response to inflammatory stimuli.

Numerous chemokine genes have been identified in teleost species and they exert a vast array of functions yet not fully elucidated. In addition to their immunological function, chemokines have a role in organogenesis and have been demonstrated to regulate homing, maturation and even micro-environmental segregation within lymphoid organs (Alejo and Tafalla 2011 for a review). Herein, at least ten chemokines were found showing different trends in expression, however they are all characterized by moderate/high transcript levels even at early stages thus supporting the hypothesis that at least some of these molecules may have an additional role in larval development.

Acquired immune elements

All jawed vertebrates possess the genetic elements essential for the functioning of adaptive immune responses (Rauta et al. 2012). The hallmark of the adaptive immunity is the ability to mount a highly specific response against virtually any invading organism. This specificity is mainly determined by B or T lymphocytes and requires a number of key molecules expressed on effector leukocytes and target cells, such as the T cell receptor (TCR), its accessory molecules CD4 and CD8, and Class I or Class II major histocompatibility complex (MHC) molecules.

Class I and class II MHC play a pivotal role in mediating antigen recognition by lymphocytes through the interaction with TCR molecules. Until now, MHC genes have been isolated and characterized in various fish species, including zebrafish (*D. rerio*), rainbow trout, channel catfish, turbot, Nile tilapia (*O. niloticus*), sea bass and halfsmooth tongue sole (*C. semilaevis*) (Pang et al. 2013).

In the present study, we identified and characterized three transcripts coding Class I MHC α chain, as well as two transcripts coding MHCIIα and three MHCIIβ molecules. SsoMHCs all share the main features conserved across vertebrates and phylogenetic analysis confirmed their close relationships with those of teleost belonging to the order Pleuronectiformes.

Antigens bound to either class I or class II MHC proteins are then recognized by lymphocytes through the TCR. Little is known about the development of T-cells and the expression of T-cell markers

during development in Solenidae, and the information is restricted to a few species in teleost (Picchietti et al. 2009, Øvergard et al. 2011). In the present study three genes important in T-cell differentiation, TCRα, TCRβ and CD3ε, were identified and their expression assessed during *S. solea* development.

SsoMHC and SsoTCR transcripts differ in their expression from those of the innate immune system being barely detectable soon after hatching and showing a marked increase at later stages. This is not unexpected since it was already reported in other teleost species that the major immune events leading to immunocompetence coincide with metamorphosis and with the larvae to juvenile transitory phase (Dios et al. 2010, Picchietti et al. 2015). This is consistent with the timing of the main lymphoid organs appearance. To date, no reports can be found in literature upon the ontogenetic development of *S. solea* lymphoid organs, while a few information upon the appearance of hematopoietic cells in immune organs is available for the closely related species Senegalese sole (*Solea senegalensis*, Cunha et al. 2008). The timing at which the haematopoietic elements could be seen coincided with the development of the thymus and the start of exogenous feeding, suggesting that the same can reasonably happens in the common sole.

There is increasing evidence demonstrating a cross-talk between neuroendocrine and immune system. In teleost fish numerous studies already revealed an important immunomodulatory role of GH/IGFI axis (Welniak et al. 2002, Yada 2007, Franz et al. 2016), in particular during thymus and thymocytes development, through both endocrine and paracrine mechanism. In higher vertebrates it has been also demonstrated that thyroid hormones (THs) modulate specific immune responses, including cell-mediated immunity, lymphocyte differentiations and proliferation, and natural killer cell activity (Hodkinson et al. 2009), while thyrotropin (TSH) acts as a growth factor for developing T cells (van der Weerd et al. 2014). Although there is scarce information about a possible role of THs in fish immune system, first evidence appear to prove that immune organs and cells are responsive to THs (Quesada-Garcia et al. 2014) and that a relationship between thyroid and thymus development and lymphopoiesis is conserved even in teleost (Lam et al. 2005).

In *S. solea* the ontogeny during larval development of both GH/IGFI axis and TH cascade was previously reported (Ferraresso et al. 2013) and their expression was proven to be concurrent with larval organogenesis and the onset of metamorphosis. In addition, temporal regulation of their transcript levels is coherent with a stimulatory role of thymocytes as deduced by looking at SsoTCR and SsoCD3ε gene expression profiles. Taken together these results might provide indirect evidence of a hormonal modulation of adaptive immune system in *S. solea.*

It is worth of notice that SsoTCRβ expression is detectable earlier than TCRα proving that *S. solea* TCRβ is the first to be rearranged and expressed on the surface of thymocytes, as observed in other teleost species (Øvergard et al. 2011).

In the present study, neither RAG1 (Recombination-activating 1), that plays an essential role in TCR locus rearrangement, nor CD4 and CD8, key markers of T-cell differentiation, were found.

The onset of CD4 and/or CD8 positive cells is correlated to the maturation of the thymus, and appear notably later compared to the appearance of double negative cells. In addition, it was already reported that CD4 and CD8 expression is delayed in comparison with RAG1 and TCR genes (e.g. ~25 days in *D. labrax*, Picchietti et al. 2009). In this context, the failure to identify/detect CD4 and CD8 transcripts might suggest that the developmental stages analyzed were too early for T-cells having undergone a full maturation. In addition, it should be taken into account that whole larvae were employed for transcriptome sequencing, thus making more difficult the detection of tissue- or cell-specific genes.

5. CONCLUSIONS

Young fish use innate mechanisms during the first weeks of their development. In this respect, ontogenetic studies are important to understand pathways appearance and to defend farmed fish against pathogens at early age. The present study provides a temporal comparative overview on the transcriptional onset of most major proteins of innate and adaptive immunity during early development of *S. solea*, a commercially important species.

We demonstrated that the main players of the innate immunity are conserved and already expressed at first larval stages providing protection from environmental pathogens even at early development. Many of these genes show higher transcript levels at hatching, leading to the hypothesis that they might play a role during embryogenesis, and their expression increase dramatically as the needs for antimicrobial defense grow (e.g. start of exogenous feeding).

Adaptive immune transcripts differ in their expression from those of the innate immune system, their onset coincides with metamorphosis and larvae to juvenile transition, and likely overlaps with the appearance and maturation of the main lymphoid organs. However, data collected suggest that at the end of metamorphosis *S. solea* cell-mediated immune system hasn’t still undergone full maturation.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIAL CAPTIONS

Supplementary file 1. List of immune-related genes identified in *S. solea* larval transcriptome

Supplementary file 2. Multiple alignment of teleost CTSL (Figure S2.1) and CRP (Figure S2.2) genes.

Supplementary file 3. Phylogenetic analysis of teleost MHCIα (Figure S3.1), MHCIIα (Figure S3.2) and MHCIIβ (Figure S3.3) genes.