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Transcriptome analysis of larval immune defence in the lamprey *Lethenteron japonicum*

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ABSTRACT

The lamprey is a primitive jawless vertebrate that occupies a critical phylogenetic position, and its larval stage represents the major portion of its life cycle [1]. Lamprey larvae have been proven to be an important model organism for studying numerous biological problems, such as the immune system, due to their unique biological features [2]. In addition, early-stage larvae have never been obtained from the wild [3]; therefore, it is necessary to establish artificial breeding of lampreys in the laboratory. However, during early development, the larvae exhibit susceptibility to saprolegniasis, and the immune responses of lamprey larvae to this infection remain poorly understood. Here, we established a model of fungal infection in lamprey larvae and then used RNA sequencing to investigate the transcript profiles of lamprey larvae and their immune responses to *Saprolegnia ferax*. Among the profiled molecules, genes involved in pathogen recognition, inflammation, phagocytosis, lysosomal degradation, soluble humoral effectors, and lymphocyte development were significantly upregulated. The results were validated by analysis of several genes by quantitative real-time PCR and whole-mount *in situ* hybridization. Finally, we performed a Western blot for VLRs in infected and uninfected lampreys. This work not only provides an animal model for studying fungal infection but also suggests a molecular basis for developing defensive strategies to manage *Saprolegnia ferax* infection.

1. Introduction

Lampreys are cyclostomes, snake-shaped animals that spend a major part of their life as larvae [1,2], and they have been considered as an excellent model to investigate developmental biology [3], vertebrate evolution [4], and especially immunity [5,6]. The adaptive immune system of lampreys is based on different isotypes of variable lymphocyte receptors (VLRs) that are expressed by distinct lymphocyte lineages [7,8], akin to the T and B lymphocytes of jawed vertebrates. VLRA and VLRC are expressed by two T-like cell lineages [9], whereas VLRB is expressed by B-like lineage counterparts [8]. Similar to the T-cell receptors and antibodies of jawed vertebrates, the vast repertoire of structurally diverse VLRs is generated by a combinatorial assembly process resembling gene conversion, which is believed to be mediated by cytidine deaminases (CDA) that are expressed in a lineage-specific manner; — CDA1 in the T-like lymphocytes and CDA2 in the B-like cell lineages [9].

The abovementioned studies were all based on the use of wild-caught larvae [3], but the limitations of this approach are obvious, particularly because the deterioration of the ecological environment made the lamprey an endangered species. Thus, the establishment of artificial breeding of lampreys in the laboratory is urgently needed, not only for the sake of this excellent model but also for the effective protection of this valuable and endangered species.

However, at the early stage of larval development, the larvae often suffer from fungal challenge [10], and one of the most devastating pathogens of larvae is *Saprolegnia ferax*, an oomycete of aquatic origin that causes significant losses to aquatic ecosystems and aquaculture worldwide [11]. This fungus causes characteristic lesions in the mouths, eyes, and gills of infected lamprey larvae, subsequently spreading throughout the body to result in severe systemic infection and finally death. The molecular mechanism of antifungal immunity in this unique species has rarely been studied.

Transcriptome sequencing (RNA-Seq) is widely used for the global

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assessment of molecular host-pathogen interactions and functional gene identification [12–15]. Transcriptome studies have significantly enhanced our understanding of the molecular mechanisms of immune responses and the genetic responses of hosts to pathogens. Here, we used RNA-Seq to analyse the molecular changes that occur during lamprey infection with *Saprolegnia ferax* to provide the first comprehensive view of the immune defence mechanisms of this species.

2. Materials and methods

2.1. Artificial breeding of Lethenteron japonicum and treatment

Lethenteron japonicum larvae were collected via artificial breeding of lamprey in our laboratory. Spawning males and females were captured from the Wusuli River with the permission of the local authorities and cultured in the laboratory. Eggs were obtained by manually squeezing the abdomens of gravid females and then deposited into a crystallization dish. Sperm from a mature male was expressed onto the eggs. Zygotes were washed with several changes of filtered water and then transferred to modified culture media (25 mM NaCl, 0.3 mM KCl, 0.2 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM HEPES, 0.01 mM EDTA) in an 18 °C incubator for long-term culture.

When the embryos entered the larval stage, we successfully established the long-term culture of the larvae. The rearing conditions were determined as follows: the appropriate light intensity is 600 lux, the suitable water temperature is 18 °C, and the suitable weaning foods for the larvae are chlorella, paramecium, and yeast. *Lethenteron japonicum* larvae were soaked in methylene blue to prevent infection.

The strain of *Saprolegnia ferax* used in this experiment was originally isolated in our laboratory from the skin lesions of a *Lethenteron japonicum* which had naturally developed saprolegniasis in the Wusuli River. The skin pieces infected by fungi were aseptically sampled using a sterile forceps and scalpel blade and subsequently placed on an agar plate containing potato dextrose agar (PDA) broth [16,17]. The inoculated plate was incubated at 20 °C for approximately 24 h until the emergence of hyphae. Then, an agar block of fully developed *Saprolegnia* hyphae was inoculated on a PDA plate and cultivated at 20 °C until the PDA plate was completely covered by fungal hyphae [18]. Sterile water was introduced into the plate to wash out the released secondary zoospores. The resultant fluid was collected and passed through a sterile surgical gauze for hyphae removal. The spore suspensions were quantified by a haemocytometer and adjusted to a concentration of 6×10^3 spores/mL [19,20]. Then, the spores were stored at 4 °C for later use.

Lethenteron japonicum larvae (20 days) were infected with *Saprolegnia ferax* or co-cultured with normal saline (NS, control) for 24 h, and then the samples were preserved in RNAlater (Invitrogen, Carlsbad, CA, USA) and kept frozen at −80 °C until use.

2.2. RNA isolation, library preparation, and sequencing

Three biological replicates were prepared for RNA sequencing (three infected groups and three uninfected groups). Total RNA was extracted using the TRIzol reagent (Ambion, Austin, TX, USA) and quantified via a NanoDrop 2000 spectrophotometer to ensure the use of sufficient RNA. The NEBNext Ultra RNA Library Prep Kit for Illumina was used to prepare the libraries, which were quantified using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The libraries were sequenced on an Illumina HiSeq 2000 to obtain 250-300 base pair (bp) paired-end reads after purification.

2.3. De novo assembly and transcriptome annotation

After removing the adapters, low-quality sequences, and reads with unknown bases (N) > 5%, the remaining clean reads were used for *de novo* assembly using Trinity software. For functional annotation analysis, all unigenes were analysed by Blastn with the NCBI nucleotide sequence (Nt) database and by Blastx with the following protein databases: NCBI non-redundant protein sequences (Nr), euKaryotic Orthologous Groups (KOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG). In addition, Gene Ontology (GO) annotations were obtained with Blast2GO.

2.4. Analysis of differential gene expression

Expression data were determined by mapping the transcriptome assembly to the genome sequence. The fragments per kilobase per million (FPKM) value was used to calculate the gene expression levels for each sample. Differentially expressed transcripts were identified using the program EdgeR. To ensure a high-quality analysis of differentially expressed genes (DEGs), the corrected q-value was used, and unigenes with a $|\log_2 \text{Ratio}| > 1$ and a q-value < 0.05 were selected as the DEGs.

2.5. Quantitative real-time PCR (qPCR)

Six DEGs were chosen for validation of the RNA-Seq results. Total RNA was isolated using the TRIzol reagent, and cDNA was transcribed using SuperScript III reverse transcriptase (Invitrogen). The validation was performed using qPCR in a LightCycler 1.5 (Roche, Basel, Switzerland) and the primers listed in Table 1. Expression levels were normalized to the GAPDH transcript, and the differences between treatments were determined by a *t*-test.

2.6. Whole-mount in situ hybridization (WISH)

Five DEGs in the larvae were chosen for the validation by WISH. Digoxygenin-labelled probes were generated with the DIG RNA Labeling Mix (Roche) and used for larval WISH as described previously [21,22]. The primers are listed in Table 1. The larvae were imaged using a Zeiss Discovery 2.0 microscope with Axiovision Rel 4.8 software.

2.7. Western blot (WB)

Whole larvae were lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% protease inhibitor cocktail (Thermo Fisher Scientific). The protein concentrations were measured using a BCA kit (Pierce, Rockford, IL, USA). A total of 20 μg of protein mixture per sample was separated on a 12% SDS-PAGE gel. Proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) and further incubated with the appropriate antibodies. Membranes were probed using the indicated antibodies against VLRA (1:2,000), VLRB (1:5,000), VLRC (1:2,000), and GAPDH (1:5,000), followed by the HRP-conjugated second antibody (Cell Signaling Technology, Danvers, MA, USA. 1:10,000). Bands were revealed with an Immobilon ECL kit (Merck Millipore, Billerica, MA, USA) and recorded on X-ray films (Kodak, Xiamen, China).

3. Results

3.1. Artificial breeding of Japanese lamprey, Lethenteron japonicum

To establish a lamprey larva model of fungal infection, we first established the artificial breeding of Japanese lamprey in our laboratory. The timing of different developmental stage series and morphological features was observed and described as shown in Fig. S1. When the embryos developed to the larval stage (20 days), the larvae were characterized by their translucent body; melanophores appeared in the head and trunk regions, and the larvae began swimming (Fig. 1).

Table 1
Primers for qPCR and WISH.

Gene		Primer sequence 5′-3′	Accession no.	Base pairs (bp)	Purpose
Blnk	F	CAAACCGAAGGGTTACCCGA	KF692036.1	154	qPCR
	R	CTCTTGGTGAGACGTGTGCT			
C3	F	GGCTCCCTCAAGCAAATCCT	AB377282.1	102	qPCR
	R	CCATCAAACCAACCCAAGCG			
Fyn	F	TGAAGAGCAAGCAAAAGCAGTCTCC	KM255114.1	157	qPCR
	R	AAGTCGGGAATGGCGTTGGTGATG			
Gapdh	F	GGCATCCAAGGGTGAACTGA	KU041137.1	127	qPCR
	R	CTGCTTGTTGAGAGCGATGC			
HMGB2	F	TGGGTAAAGGAGAGCCAGGAAAACC	HQ615992.1	115	qPCR
	R	GCAAAGTTTACAGATGCCTCCGGA			
Intelectin B	F	AAGTTGCGATGGGGGTTTTGTTACC	AB114629.1	120	qPCR
	R	GCAATTTGAACAGCCCCATGGA			
Notch	F	GACCAGCCCAATGCCTACTT	AB292627.1	199	qPCR
	R	GGCTGCTCTGGCATTCATTG			
C3	F	GCACTTTTGGTGACGGTGTC	AB377282.1	473	WISH
	R	CACAAACGCTGTCAACCAGG			
Notch	F	AATGCCCTGTCTCAACGACG	AB292627.1	503	WISH
	R	GGTAGCGGTTCACCAGATCC			
VLRA	F	ATCATCCTCATCCTCCTCCTCATCG	AB507269.1	478	WISH
	R	TTGCGTAGAGAAGGATCGTCTGCAG			
VLRB	F	GCCTGCAGGAGCCAATCATCATGT	AB507270.1	496	WISH
	R	CAGTCCCAGGGGTTGTTGAACAAC			
VLRC	F	AGCTAACGTCTCTCCCACCT	AB507271.1	487	WISH
	R	TGCGTTCTGTGCTCATGGAT			

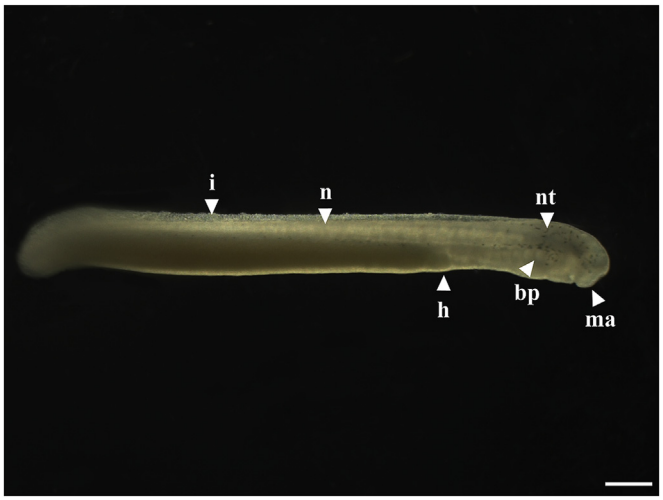

Fig. 1. Larva of *Lethenteron japonicum*. bp, branchial pore; h, heart; i, integument; ma, mandibular arch; n, notochord; nt, neural tube. Bar, 200 μm.

3.2. Transcriptome sequencing and assembly

Using the Illumina HiSeq 2000 sequencer, we generated 55.69 Gb of sequencing data from the six samples, with an average of 92.8 million reads per sample and a read length of 250–300 bp. In total, 512,878 transcripts were predicted from the clean reads. After redundancy was removed, 339,967 unigenes with an average length of 1232 bp were assembled. Among these unigenes, 125,369 were 200–500 bp in length, 93,890 were 500–1000 bp, 60,895 were 1000–2000 bp and 59,813 were longer than 2000 bp (Table 2).

3.3. Functional annotation

To assign potential functions to the *Lethenteron japonicum* larval transcriptome, 339,967 unigenes were subjected to annotation analysis. In total, 116,648 (34.25%) were found in Nr, 151,063 (44.43%) were found in Nt, 45,176 (13.28%) were found in KOG, 61,356 (18.04%) were found in KEGG, 97,588 (28.7%) were found in Swiss-Prot, 127,134 (33.39%) were found in Pfam and 127,557 (37.52%) were found in the GO database.

Table 2
Length distributions of assembled transcripts and unigenes.

Length range	Number of transcripts (ratio, %)	Number of unigenes (ratio, %)
200-500 bp	291,488 (70.37%)	125,369
500-1000 bp	100,361 (14.68%)	93,890
1000-2000 bp	61,195 (8.43%)	60,895
> 2000 bp	59,834 (6.51%)	59,813
Total	512,878	339,967
Total length (bp)	470,415,931	418,705,050
N50 length (bp)	1843	2194
Mean length (bp)	917	1232

The functional properties of genes and gene products can be comprehensively analysed by GO. A total of 127,557 unigenes were successfully classified into three major categories: molecular function (10 terms), cellular component (20 terms) and biological process (26 terms) (Fig. 2). Within the biological process category, the most abundant transcripts were assigned to “cellular process” and “metabolic process”. For the cellular component, the most highly represented term was “cell”, followed by “cell part”. “Binding” was the most abundant GO term for molecular function (Fig. 2).

KOG classification of unigenes is essential for evolutionary studies and functional annotation. Here, 45,176 genes were successfully annotated into 26 functional categories. The most abundant cluster was “signal transduction mechanisms” (Fig. 3).

Furthermore, KEGG pathway analysis was further carried out to identify the biological processes. In total, 64,887 genes were assigned into 32 subcategory pathways. The largest subcategory group, signal transduction, had 9,891 annotated genes, followed by endocrine system (4,973), cellular community (4,159), transport and catabolism (3,740), and immune system (3,662) (Fig. 4).

3.4. Differentially expressed genes

Overall, 10,034 DEGs were detected, of which 3,136 and 6,898 were down- and upregulated, respectively (up-/downregulated DEGs ratio of approximately 2.2/1). These unigenes may be related to *Saprolegnia ferax* infection (Fig. 5).

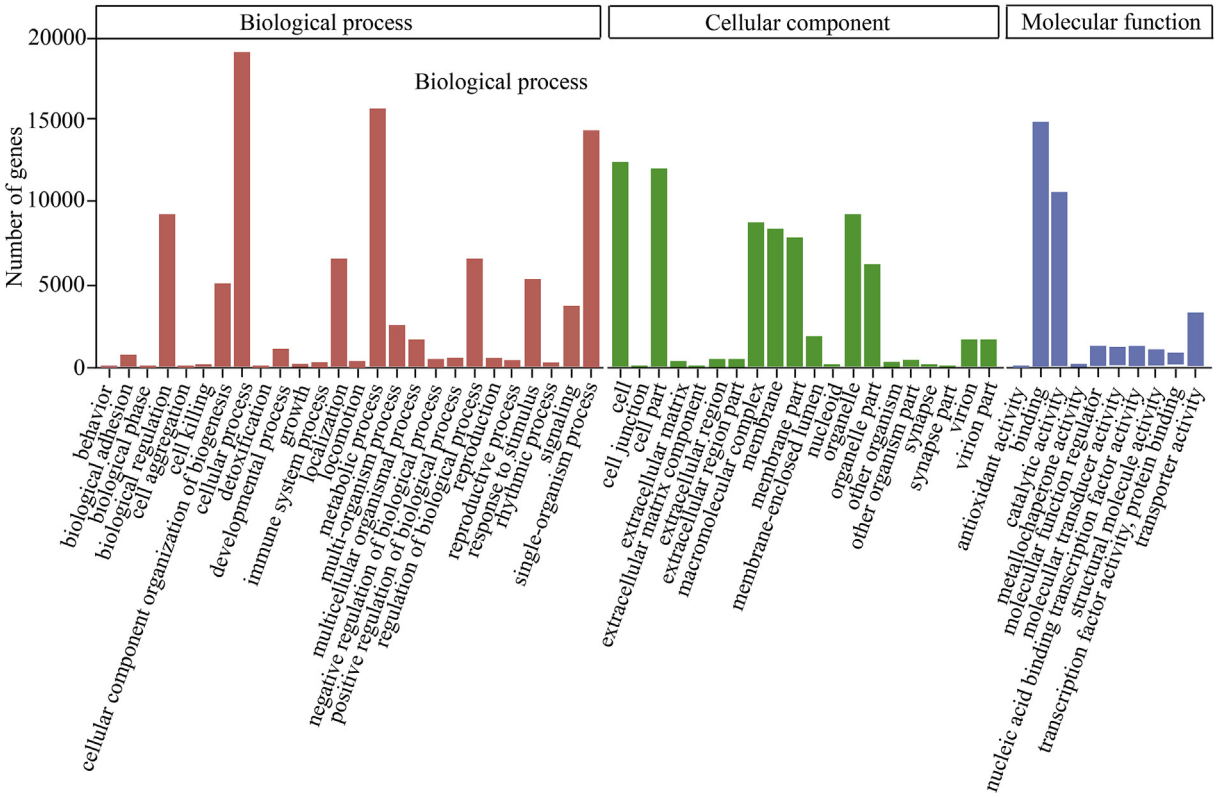

Fig. 2. Gene Ontology (GO) classifications of non-redundant unigenes. All annotated unigenes were categorized into 3 categories: A: biological process, B: cellular component, C: molecular function.

3.5. Functional enrichment analysis of DEGs

To further understand the functions of DEGs associated with *Saprolegnia ferax* infection, analyses of some immune-related pathways were performed (Table 3), such as the apoptosis [23], Toll-like receptor [24] and NF-kB signalling pathways [25]. The NF-kB signalling pathway is a critical player in Toll-like receptor signalling, regulating immune system responses, inflammation factors, and apoptosis. Apoptosis acts as a mediator of immune defence against various pathogens in both vertebrates and invertebrates [26].

The pathway with the greatest representation by unique genes was the PI3K-Akt signalling pathway (ko04151) [27], with 121 members. In this pathway, Toll-like receptors (TLR2/4), phosphatidylinositol 3 kinase (PI3K), protein phosphatase (PP2A), insulin receptor substrate (IRS1), protein kinase C (PKCs), and AMP-activated protein kinase (AMPK) were associated with the upregulated DEGs. These genes play vital roles in immune responses. Focal adhesion kinase (FAK) and a proto-oncogene (Ras) were associated with downregulated DEGs. Proto-oncogenes (c-Myb) were also associated with up- and down-regulated DEGs (Fig. S2). The PI3K-Akt signalling pathway not only plays vital roles in regulating adaptive immune cell activation but is also involved in many important pathological and physiological processes of innate immunity. These observation may suggest that the immune system response of lamprey larvae to fungal infection may be

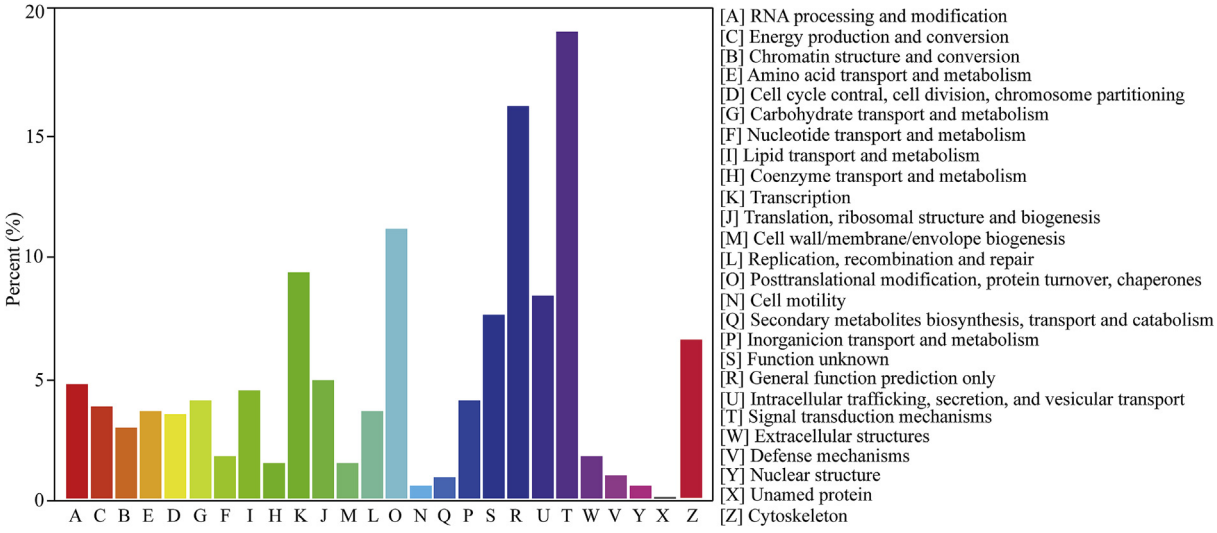

Fig. 3. Clusters of orthologous groups (KOG) classification of putative proteins.

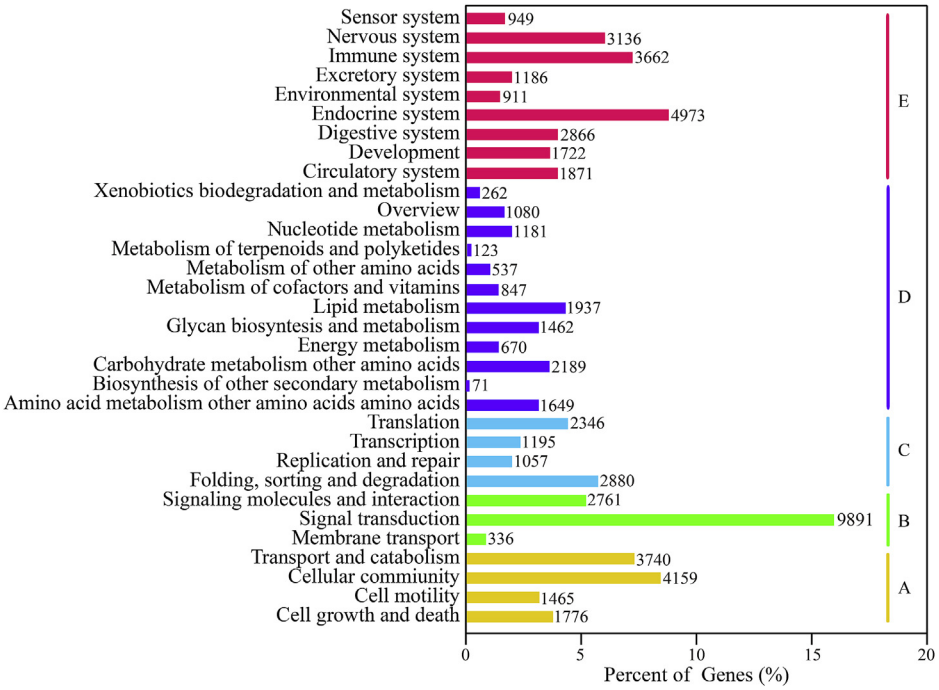

Fig. 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) classifications of non-redundant unigenes. The Y-axis indicates specific gene categories. The X-axis indicates the number of genes in a category. A, Cellular Processes; B, Environmental Information Processing; C, Genetic Information Processing; D, Metabolism; E, Organismal Systems.

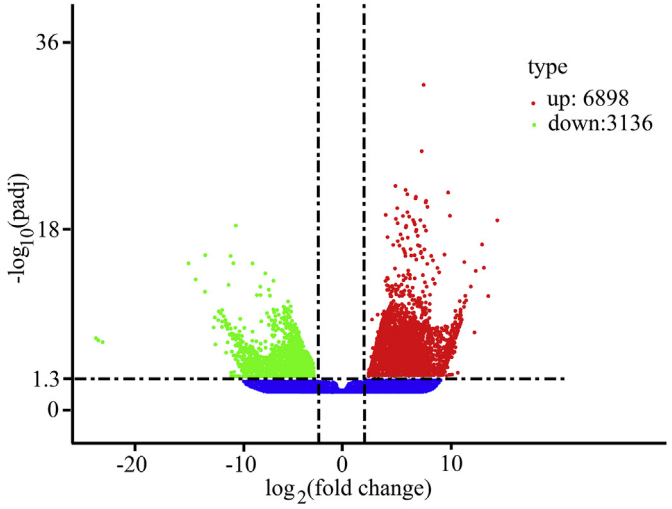

Fig. 5. Volcano plot of differentially expressed genes. The X-axis represents the fold change, while the Y-axis indicates the significance of differential expression. The blue dots signify no significant changes in the unigenes, while the red and green dots signify up- and downregulated unigenes, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diverse and complex.

3.6. Analysis of differentially expressed immune-related genes

According to the DEG annotation information, a large proportion of the significantly identified DEGs were associated with host immune responses. Many immune-related genes were changes in *Saprolegnia ferax*-infected larvae (Table 4). Upregulated genes included pathogen recognition receptors (TLR2) [28], antimicrobial factors (lysozyme) [29], inflammation (IL-17R, SAA) [30–32], interferon regulatory factor (IRF4/8) [33–35], and several apoptosis-related transcripts (bcl-2) [36]. Upregulated transcripts also included lymphocyte development (Notch) [37–39] and lymphocyte transcription factors (c-Rel) [40,41] (Table 4). Downregulated transcripts in the infected larvae included soluble humoral effectors (C3) [42], intelectin B [43], a DNA binding protein (HMGB2) [44–46] and a DNA repair molecule (Artemis) [47] (Table 4). These DEGs may play a vital role in the elimination of externally infecting pathogens.

Table 3
KEGG classifications of differentially expressed genes involved in immune-related pathways.

KEGG pathways	Number of differentlly expressed genes	Pathway ID
PI3K-Akt signalling pathway	121	ko04151
MAPK signalling pathway	90	ko04010
Ras signalling pathway	78	ko04014
Apoptosis	70	ko04210
Lysosome	60	ko04142
Phagosome	60	ko04145
FoxO signalling pathway	55	ko04068
Platelet activation	42	ko04611
TGF-beta signalling pathway	36	ko04350
Chemokine signalling pathway	34	ko04062
NF-kappa B signalling pathway	33	ko04064
Fc gamma R-mediated phagocytosis	30	ko04666
TNF signalling pathway	28	ko04668
Antigen processing and presentation	23	ko04612
Hippo signalling pathway	21	ko04391
Natural killer cell mediated cytotoxicity	19	ko04650
T cell receptor signalling pathway	19	ko04660
B cell receptor signalling pathway	17	ko04662
mTOR signalling pathway	15	ko04150
Toll-like receptor signalling pathway	14	ko04620
RIG-I-like receptor signalling pathway	13	ko04622
Jak-STAT signalling pathway	6	ko04630

3.7. Confirmation of DEG expression by qPCR

To better understand the immune response of lamprey larvae to *Saprolegnia ferax* and to confirm the transcriptome data, qPCR analysis

Table 4
Immune-related differentially expressed genes after *Saprolegnia ferax* infection.

Unigenes	Gene symbol	Log_2Ratio
Cluster-34530.170526	Interferon regulatory factor 8 (IRF8)	8.6
Cluster-34530.54914	Lysozyme	8.5
Cluster-34530.215204	Lymphocyte cell kinase (Lck)	7.7
Cluster-34530.71168	Toll-like receptor 2 (TLR2)	6.6
Cluster-34530.193007	Galectin 8	6.5
Cluster-34530.244907	Notch	6.0
Cluster-34530.12495	Toll-like receptor 1 (TLR1)	5.3
Cluster-34530.262741	FYN proto-oncogene (Fyn)	4.8
Cluster-34530.256912	Serum amyloid A (SAA)	4.8
Cluster-34530.15319	B-cell linker protein (Blnk)	4.7
Cluster-34530.47832	Toll-like receptor 13 (TLR13)	4.2
Cluster-34530.202307	Interferon regulatory factor 4 (IRF4)	3.2
Cluster-34530.191925	Interleukin-17 receptor (IL-17R)	2.8
Cluster-34530.187431	Proto-oncogene c-Rel (c-Rel)	2.4
Cluster-34530.126364	B-cell lymphoma-2 (Bcl-2)	1.5
Cluster-34530.137586	High mobility group protein B2 (HMGB2)	−2.3
Cluster-34530.138400	Galectin 3	−2.7
Cluster-34530.4267	Intelectin B	−2.9
Cluster-34530.144821	Complement component 3 (C3)	−4.8
Cluster-34530.96132	Artemis	−7.9

was performed on a number of genes related to the immune system. The selected DEGs were categorized with KEGG pathway enrichment analyses, such as the PI3K-Akt signalling pathway, phagosome, and Toll-like receptor signalling. These genes included the FYN proto-oncogene (*Fyn*) [48], *Notch*, B-cell linker protein (*Blnk*) [49], high mobility group protein B2 (*HMGB2*), *intelectin B*, and complement component 3 (*C3*) [50,51].

The qPCR results showed significant upregulation in the expression of *Fyn*, *Notch*, and *Blnk* after challenge with *Saprolegnia ferax* (Fig. 6). However, the expression of *HMGB2*, *intelectin B*, and *C3* was downregulated in infected larvae. In short, the expression patterns of all observed genes showed the same trends between the transcriptome and real-time PCR analysis (Fig. 6).

3.8. WISH and immunoblot analysis

WISH was also used to investigate the immune responses of larvae to *Saprolegnia ferax*. *In situ* hybridization at the larval stage (20 days) showed that the mRNA level of *Notch* was upregulated and that of *C3* was downregulated (Fig. 7). However, no change in the expression levels of *VLRA*, *VLRB*, and *VLRC* was observed between diseased larvae and healthy individuals, and these data were consistent with the subsequent immunoblot analysis (Fig. 8). There were no obvious differences in antigen receptor (VLRA, VLRB, VLRC) expression between lamprey larvae before and after *Saprolegnia ferax* treatment.

In addition, the expression patterns of the *Notch* and *C3* genes were also revealed by *in situ* hybridization. *Notch* expression was observed in the notochord, branchial pore, and neural tube (Fig. 7B, B'), while *C3* was expressed in the mandibular arch, notochord, and neural tube (Fig. 7C, C'). These results indicate that these genes indeed play essential roles in the immune response of larvae to the challenge with *Saprolegnia ferax*.

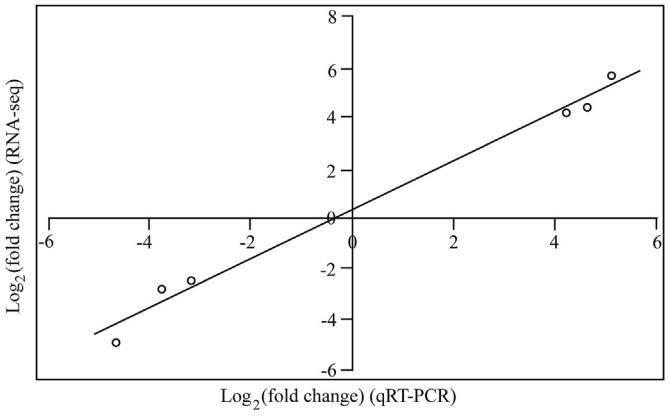

Fig. 6. Validation of differentially expressed genes (DEGs) by qPCR. To validate the RNA-seq data, the relative gene expression levels of 6 selected DEGs, FYN proto-oncogene (*Fyn*), *Notch*, B-cell linker protein (*Blnk*), high mobility group protein B2 (*HMGB2*), *intelectin B*, and complement component 3 (*C3*), were examined by qPCR.

4. Discussion

Lampreys are pivotal representatives for studying the immune system due to their unique position in chordate phylogeny. However, during early development, lamprey larvae often suffer from fungal infection, and the molecular mechanism of antifungal immunity in this unique species remains poorly understood. In this study, the primary objective was to investigate molecular changes associated with saprolegniasis in the artificial breeding and rearing of lamprey larvae. Thus, we used RNA-seq to sequence the transcriptomes of larvae with *Saprolegnia ferax* infection to provide the first comprehensive view of the genes transcribed in response to this fungus.

A number of differentially expressed genes (DEGs) related to immune pathways were observed in *Lethenteron japonicum* larvae during the stages of *Saprolegnia ferax* infection. These DEGs mediate a broad range of immune functions, such as innate immune recognition (lectins), apoptosis regulation (bcl-2), and defence (lysozyme). Other immune-related DEGs may directly support the function and development of lymphocytes that mediate adaptive immunity, including genes for lymphocyte transcription factors (c-Rel), lymphocyte signalling (CD45), and genes expressed by epithelial cells (DLL-B).

Upregulated transcripts in the infected larvae included *Fyn*, *Blnk* and *Notch*. Notch was initially shown to be a key determinant of cell-lineage commitment in developing lymphocytes [52], but it is now known to mediate the innate immune response by regulating TLRs, lectins and complement [38,53–56]. Fyn, a member of the Src family kinases, plays a significant role in apoptosis and immune response [57–59]. As principal components of the adaptive immune system, B lymphocytes (B cells) perform various immune functions, such as producing different cytokines; Blnk is an adaptor protein that plays a crucial role in the B cell antigen receptor (BCR) signalling pathway [60,61]. These genes may contribute to the immune response of *Lethenteron japonicum* to *Saprolegnia ferax* infection.

In contrast, the downregulated genes included *HMGB2*, *intelectin B*, and *C3*. C3 is central to the complement system, which has a major role in innate immunity [50]. The intelectin family is a group of secretory lectins that serve multiple functions [43], including innate immunity [62,63]. HMGB was previously thought to function only as a nuclear factor that enhances transcription [64], but it was recently shown to also participate in the response to infection, injury and inflammation [65]. Notably, the variable lymphocyte receptors (VLRA, VLRB, VLRC) [66], which are vital for adaptive immunity in lampreys, were not found among the DEGs [67,68], implying that the lamprey larva mainly relies on its innate immunity rather than the adaptive immune system to defend against pathogens at the early stage of larval development. This hypothesis may explain why these larvae are susceptible to saprolegniasis.

The results also showed that different transcripts belonging to the same functional group sometimes displayed opposing trends, such as C1q, defensin, and cathepsin. C1q proteins have been previously shown to represent a highly diverse group of molecules playing an important role as pathogen recognition receptors [69]. Our results also identified several defensins which were present among transcripts upregulated in infected larvae while other members of the same group were downregulated. Defensins are a large group of small antimicrobial peptides

Fig. 7. Expression of immune-related genes in lamprey larvae. Gene-specific expression, as determined by RNA *in situ* hybridization with gene-specific riboprobes, is shown in blue. (A, A′) Control analysis of diseased larvae (A) and healthy individuals (A′). (B, B′) Notch expression in diseased larvae (B) and healthy individuals (B′). (C, C′) C3 expression in diseased larvae (C) and healthy individuals (C′). (D, D′) VLRA expression in diseased larvae (D) and healthy individuals (D′). (E, E′) VLRB expression in diseased larvae (E) and healthy individuals (E′). (F, F′) VLRC expression in diseased larvae (F) and healthy individuals (F′). Bar, 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

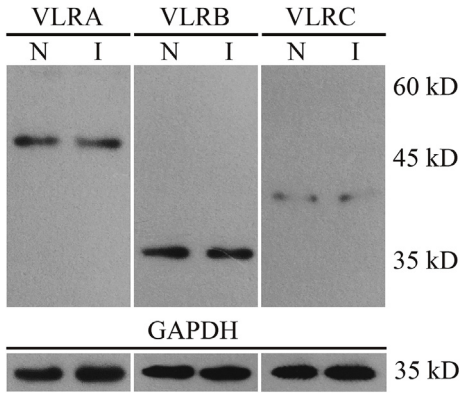

Fig. 8. Western blot (WB) analysis of larvae before and after *Saprolegnia ferax* exposure. Gene-specific expression as determined by WB with VLRA-, VLRB-, and VLRC-specific antibodies. N, naive; I, immunized. kDa, kilodaltons.

and represent major actors in innate immunity. Defensins from the scallop *Argopecten irradians* and the horseshoe crab *Tachypleus tridentatus* exhibit strong fungicidal activities [70,71]. Another group of transcripts highly regulated in *Saprolegnia ferax*-infected larvae was cathepsins. As members of the cysteine proteases, cathepsins are also strongly implicated in the regulation of apoptosis, and changes in their expression were associated with the regulation of apoptosis-related proteins [72]. These findings highlight the need for further investigations to generate a better understanding of the specific molecular roles of different transcripts in response to *Saprolegnia ferax* infection.

Moreover, the morbidity and mortality associated with fungal infections, together with the emergence of drug-resistant strains, necessitate broadening our knowledge of host antifungal immune responses and fungal pathogenesis [73,74]. The unique biological features of lampreys and the successful establishment of the artificial lamprey larva model in the laboratory could help achieve these goals [75].

In conclusion, we used RNA-Seq to sequence the transcriptomes of larvae infected with *Saprolegnia ferax* to provide the first comprehensive view of gene transcription in response to this fungus. A number of genes associated with the immune defence response were identified, and significant alterations in immune-related genes were found, suggesting that they may be used to mediate a broad range of immune functions. This study may help our understanding of larval immune responses to infection. In addition, the results reported here highlight the crucial role of the innate immune system in the larval response to saprolegniasis. Nevertheless, additional work is needed to further characterize the detailed molecular mechanisms associated with the larval immune response to the infection with *Saprolegnia ferax*.

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Appendix A. Supplementary data

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References

[1] M.M. Chang, F. Wu, D. Miao, et al., Discovery of fossil lamprey larva from the Lower Cretaceous reveals its three-phased life cycle, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 15486–15490.
[2] B. Bajoghli, P. Guo, N. Aghaallaei, et al., A thymus candidate in lampreys, Nature 470 (2011) 90–94.
[3] S.M. Shimeld, P.C. Donoghue, Evolutionary crossroads in developmental biology: cyclostomes (lamprey and hagfish), Development 139 (2012) 2091–2099.
[4] M.D. Cooper, M.N. Alder, The evolution of adaptive immune systems, Cell 124 (2006) 815–822.
[5] Z. Pancer, W.E. Mayer, J. Klein, et al., Prototypic T cell receptor and CD4-like coreceptor are expressed by lymphocytes in the agnathan sea lamprey, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 13273–13278.
[6] M. Hirano, P. Guo, N. McCurley, et al., Evolutionary implications of a third lymphocyte lineage in lampreys, Nature 501 (2013) 435–438.
[7] G.W. Litman, J.P. Rast, S.D. Fugmann, The origins of vertebrate adaptive immunity, Nat. Rev. Immunol. 10 (2010) 543–553.
[8] T. Boehm, N. McCurley, Y. Sutoh, et al., VLR-based adaptive immunity, Annu. Rev. Immunol. 30 (2012) 203–220.
[9] S.J. Holland, M. Gao, M. Hirano, et al., Selection of the lamprey VLRC antigen receptor repertoire, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 14834–14839.
[10] A. Verma, M. Wuthrich, G. Deepe, et al., Adaptive immunity to fungi, Cold Spring Harb. Perspect. Med. 5 (2014) a019612.
[11] J.M. Kiesecker, A.R. Blaustein, L.K. Belden, Complex causes of amphibian population declines, Nature 410 (2001) 681–684.
[12] B. Allam, E. Pales Espinosa, A. Tanguy, et al., Transcriptional changes in manila clam (*Ruditapes philippinarum*) in response to Brown ring disease, Fish Shellfish Immunol. 41 (2014) 2–11.
[13] T.K. Herath, J.E. Bron, K.D. Thompson, et al., Transcriptomic analysis of the host response to early stage salmonid alphavirus (SAV-1) infection in Atlantic salmon *Salmo salar* L, Fish Shellfish Immunol. 32 (2012) 796–807.
[14] R.L. Quispe, E.B. Justino, F.N. Vieira, et al., Transcriptional profiling of immune-related genes in Pacific white shrimp (*Litopenaeus vannamei*) during ontogenesis, Fish Shellfish Immunol. 58 (2016) 103–107.
[15] Y. Zhang, I. Soderhall, K. Soderhall, et al., Expression of immune-related genes in one phase of embryonic development of freshwater crayfish, *Pacifastacus leniusculus*, Fish Shellfish Immunol. 28 (2010) 649–653.
[16] J.M. Romansic, K.A. Diez, E.M. Higashi, et al., Effects of the pathogenic water mold *Saprolegnia ferax* on survival of amphibian larvae, Dis. Aquat. Org. 83 (2009) 187–193.
[17] S. Basu, C. Bose, N. Ojha, et al., Evolution of bacterial and fungal growth media, Bioinformation 11 (2015) 182–184.
[18] M.G. Andersson, L. Cerenius, Pumilio homologue from *saprolegnia* parasitica specifically expressed in undifferentiated spore cysts, Eukaryot. Cell 1 (2002) 105–111.
[19] H. Cao, W. Zheng, J. Xu, et al., Identification of an isolate of *Saprolegnia ferax* as the causal agent of saprolegniosis of yellow catfish (*Pelteobagrus fulvidraco*) eggs, Vet. Res. Commun. 36 (2012) 239–244.
[20] M.N. Sarowar, A.H. van den Berg, D. McLaggan, et al., *Saprolegnia* strains isolated from river insects and amphipods are broad spectrum pathogens, Fungal Biol. 117 (2013) 752–763.
[21] T. Jowett, L. Lettice, Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes, Trends Genet. 10 (1994) 73–74.
[22] J. Hejatko, I. Blilou, P.B. Brewer, et al., *In situ* hybridization technique for mRNA detection in whole mount Arabidopsis samples, Nat. Protoc. 1 (2006) 1939–1946.
[23] P.G. Clarke, S. Clarke, Historic apoptosis, Nature 378 (1995) 230.
[24] B. Beutler, Inferences, questions and possibilities in Toll-like receptor signalling, Nature 430 (2004) 257–263.
[25] S. Gerondakis, R. Grumont, R. Gugasyan, et al., Unravelling the complexities of the NF-kappaB signalling pathway using mouse knockout and transgenic models, Oncogene 25 (2006) 6781–6799.
[26] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495–516.
[27] T. Ersahin, N. Tuncbag, R. Cetin-Atalay, The PI3K/AKT/mTOR interactive pathway, Mol. Biosyst. 11 (2015) 1946–1954.
[28] P. Mistry, M.H. Laird, R.S. Schwarz, et al., Inhibition of TLR2 signaling by small molecule inhibitors targeting a pocket within the TLR2 TIR domain, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 5455–5460.
[29] S.A. Ragland, A.K. Criss, From bacterial killing to immune modulation: recent insights into the functions of lysozyme, PLoS Pathog. 13 (2017) e1006512.
[30] C. Gu, L. Wu, X. Li, IL-17 family: cytokines, receptors and signaling, Cytokine 64 (2013) 477–485.
[31] A. Digre, J. Nan, M. Frank, et al., Heparin interactions with apoA1 and SAA in inflammation-associated HDL, Biochem. Biophys. Res. Commun. 474 (2016) 309–314.
[32] R.H. Buckley, Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution, Annu. Rev. Immunol. 22 (2004) 625–655.
[33] H. Nguyen, J. Hiscott, P.M. Pitha, The growing family of interferon regulatory factors, Cytokine Growth Factor Rev. 8 (1997) 293–312.
[34] K. Honda, A. Takaoka, T. Taniguchi, Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors, Immunity 25 (2006) 349–360.
[35] J. Mahnke, V. Schumacher, S. Ahrens, et al., Interferon Regulatory Factor 4 controls TH1 cell effector function and metabolism, Sci. Rep. 6 (2016) 35521.
[36] P. Polcic, M. Mentel, G. Gavurnikova, et al., To keep the host alive - the role of viral Bcl-2 proteins, Acta Virol. 61 (2017) 240–251.
[37] D.A. Sultana, J.J. Bell, D.A. Zlotoff, et al., Eliciting the T cell fate with Notch, Semin. Immunol. 22 (2010) 254–260.
[38] T. Ito, J.M. Connett, S.L. Kunkel, et al., Notch system in the linkage of innate and adaptive immunity, J. Leukoc. Biol. 92 (2012) 59–65.
[39] F. Radtke, A. Wilson, S.J. Mancini, et al., Notch regulation of lymphocyte development and function, Nat. Immunol. 5 (2004) 247–253.
[40] Y. Grinberg-Bleyer, H. Oh, A. Desrichard, et al., NF-kappaB c-rel is crucial for the regulatory T cell immune checkpoint in cancer, Cell 170 (2017) 1096-108.e13.
[41] H. Zhang, J. Bi, H. Yi, et al., Silencing c-Rel in macrophages dampens Th1 and Th17 immune responses and alleviates experimental autoimmune encephalomyelitis in mice, Immunol. Cell Biol. 95 (2017) 593–600.
[42] Y. Chen, K. Xu, J. Li, et al., Molecular characterization of complement component 3 (C3) in Mytilus coruscus improves our understanding of bivalve complement system, Fish Shellfish Immunol. 76 (2018) 41–47.
[43] L. Chen, J. Yan, J. Shi, et al., Zebrafish intelectin 1 (zITLN1) plays a role in the innate immune response, Fish Shellfish Immunol. 83 (2018) 96–103.
[44] H. Yanai, T. Ban, Z. Wang, et al., HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses, Nature 462 (2009) 99–103.
[45] Y. Rao, J. Su, C. Yang, et al., Characterizations of two grass carp Ctenopharyngodon idella HMGB2 genes and potential roles in innate immunity, Dev. Comp. Immunol. 41 (2013) 164–177.
[46] D.C. Avgousti, C. Herrmann, K. Kulej, et al., A core viral protein binds host nucleosomes to sequester immune danger signals, Nature 535 (2016) 173–177.
[47] D. Moshous, I. Callebaut, R. de Chasseval, et al., Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency, Cell 105 (2001) 177–186.
[48] E. Yamada, S. Okada, C.C. Bastie, et al., Fyn phosphorylates AMPK to inhibit AMPK activity and AMP-dependent activation of autophagy, Oncotarget 7 (2016) 74612–74629.
[49] R. Pappu, A.M. Cheng, B. Li, et al., Requirement for B cell linker protein (BLNK) in B cell development, Science 286 (1999) 1949–1954.
[50] B.J. Janssen, E.G. Huizinga, H.C. Raaijmakers, et al., Structures of complement component C3 provide insights into the function and evolution of immunity, Nature 437 (2005) 505–511.
[51] Y. Kimura, N. Inoue, A. Fukui, et al., A short consensus repeat-containing complement regulatory protein of lamprey that participates in cleavage of lamprey complement 3, J. Immunol. 173 (2004) 1118–1128.
[52] P. Hertzog, A notch in the toll belt, Immunity 29 (2008) 663–665.
[53] F. Radtke, H.R. MacDonald, F. Tacchini-Cottier, Regulation of innate and adaptive immunity by Notch, Nat. Rev. Immunol. 13 (2013) 427–437.
[54] C. Ji, X. Guo, X. Dong, et al., Notch1a can widely mediate innate immune responses in zebrafish larvae infected with Vibrio parahaemolyticus, Fish Shellfish Immunol. 92 (2019) 680–689.
[55] Q. Shen, B. Cohen, W. Zheng, et al., Notch shapes the innate immunophenotype in breast cancer, Cancer Discov. 7 (2017) 1320–1335.
[56] Y. Shang, S. Smith, X. Hu, Role of Notch signaling in regulating innate immunity and inflammation in health and disease, Protein Cell 7 (2016) 159–174.
[57] Q. Zhang, X. Song, P. Su, et al., A novel homolog of protein tyrosine kinase Fyn identified in *Lampetra japonica* with roles in the immune response, Gene 579 (2016) 193–200.
[58] A.A. Nemec, L.M. Zubritsky, A. Barchowsky, Chromium(VI) stimulates Fyn to initiate innate immune gene induction in human airway epithelial cells, Chem. Res. Toxicol. 23 (2010) 396–404.
[59] C. Picard, A. Gilles, P. Pontarotti, et al., Cutting edge: recruitment of the ancestral fyn gene during emergence of the adaptive immune system, J. Immunol. 168 (2002) 2595–2598.
[60] Z.Q. Mo, J.L. Wang, R. Han, et al., Identification and functional analysis of grouper (Epinephelus coioides) B-cell linker protein BLNK, Fish Shellfish Immunol. 81 (2018) 399–407.
[61] Y. Han, X. Liu, B. Shi, et al., Identification and characterisation of the immune response properties of Lampetra japonica BLNK, Sci. Rep. 6 (2016) 25308.
[62] M. Peng, D. Niu, F. Wang, et al., Complement C3 gene: expression characterization and innate immune response in razor clam *Sinonovacula constricta*, Fish Shellfish Immunol. 55 (2016) 223–232.
[63] S. Nagata, Identification and characterization of a novel intelectin in the digestive tract of *Xenopus laevis*, Dev. Comp. Immunol. 59 (2016) 229–239.
[64] M.T. Lotze, K.J. Tracey, High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal, Nat. Rev. Immunol. 5 (2005) 331–342.
[65] N. Taniguchi, Y. Kawakami, I. Maruyama, et al., HMGB proteins and arthritis, Hum. Cell 31 (2018) 1–9.
[66] S.J. Holland, L.M. Berghuis, J.J. King, et al., Expansions, diversification, and interindividual copy number variations of AID/APOBEC family cytidine deaminase genes in lampreys, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E3211–E3220.
[67] J.P. Rast, K.M. Buckley, Lamprey immunity is far from primitive, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 5746–5747.
[68] P. Guo, M. Hirano, B.R. Herrin, et al., Dual nature of the adaptive immune system in lampreys, Nature 459 (2009) 796–801.
[69] M. Son, B. Diamond, F. Santiago-Schwarz, Fundamental role of C1q in autoimmunity and inflammation, Immunol. Res. 63 (2015) 101–106.
[70] T. Saito, S. Kawabata, T. Shigenaga, et al., A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence, and antibacterial activity, J. Biochem. 117 (1995) 1131–1137.
[71] J. Zhao, L. Song, C. Li, et al., Molecular cloning, expression of a big defensin gene from bay scallop *Argopecten irradians* and the antimicrobial activity of its

recombinant protein, Mol. Immunol. 44 (2007) 360–368.
[72] B.G. Sun, H. Chi, Cathepsin S of *Sciaenops ocellatus*: identification, transcriptional expression and enzymatic activity, Int. J. Biol. Macromol. 82 (2016) 76–82.
[73] L. Tedersoo, M. Bahram, S. Polme, et al., Fungal biogeography. Global diversity and geography of soil fungi, Science 346 (2014) 1256688.
[74] D. Hibbett, MICROBIOLOGY. The invisible dimension of fungal diversity, Science 351 (2016) 1150–1151.
[75] J. Osorio, S. Retaux, The lamprey in evolutionary studies, Dev. Gene. Evol. 218 (2008) 221–235.