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Gene expression profiles alteration after infection of virus, bacteria, and parasite in the Olive flounder (*Paralichthys olivaceus*)

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Olive flounder (*Paralichthys olivaceus*) is one of economically valuable fish species in the East Asia. In comparison with its economic importance, available genomic information of the olive flounder is very limited. The mass mortality caused by variety of pathogens (virus, bacteria and parasites) is main problem in aquaculture industry, including in olive flounder culture. In this study, we carried out transcriptome analysis using the olive flounder gill tissues after infection of three types of pathogens (Virus; Viral hemorrhagic septicemia virus, Bacteria; *Streptococcus parauberis*, and Parasite; *Miamiensis avidus*), respectively. As a result, we identified total 12,415 differentially expressed genes (DEG) from viral infection, 1,754 from bacterial infection, and 795 from parasite infection, respectively. To investigate the effects of pathogenic infection on immune response, we analyzed Gene ontology (GO) enrichment analysis with DEGs and sorted immune-related GO terms per three pathogen groups. Especially, we verified various GO terms, and genes in these terms showed down-regulated expression pattern. In addition, we identified 67 common genes (10 up-regulated and 57 down-regulated) present in three pathogen infection groups. Our goals are to provide plenty of genomic knowledge about olive flounder transcripts for further research and report genes, which were changed in their expression after specific pathogen infection.

Olive flounder is one of main marine species having high economic value in the countries of East Asia. Because of industrial importance by increasing of demand, it is serious subject to understand the pathogenic infection and production performance of olive flounder. Mass mortality of fishes is the most severe problem, accompanying vast deficit in aquaculture farm. Efforts to prevent regrettable death have especially been conducted in immunology¹⁻³. However, so far, it is hard to figure out cause of death exactly in short time because of variables of expansive marine ecosystem. Mass mortality of olive flounder was normally caused by diseases via various sources of infection such as virus, bacteria, or parasite from external environment.

Viruses depend on host cell ribosomes to produce their proteins, and sometime use host cell DNA and RNA polymerases for replication and transcription, respectively. Many viruses encode proteins that modify the host transcription or translation apparatus to favor the synthesis of viral proteins over those of the host cell. Among

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viruses, viral hemorrhagic septicemia virus (VHSV) is affiliated to *Novirhabdovirus* genus, which is a member of the *Rhabdoviridae* family⁴. The six gene were contained in the VHSV genome of about 11 K bases and each of them coded nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonstructural viral protein (NV), and RNA polymerase (L) in the following order 3'-N-P-M-G-NV-L-5'⁴. Infection of VHSV results in contagious viral hemorrhagic septicemia (VHS) in diverse fish species regardless of their inhabitation; seawater or freshwater⁵. In East Asia, a lot of infection cases into olive flounder have been reported steadily, since VHSV was detected in middle of 1990s⁶⁻⁹.

A variety of scuticociliates have been reported as cause of scuticociliatosis in marine species including turbot, guppy, and southern bluefin tuna¹⁰⁻¹². In olive flounder, disease has been reported to be causing from various scuticociliates; *Uronema marinum*, *Pseudocohnilembus persalinus*, *Philasterides dicentrarchi*, *Miamiensis avidus*¹³⁻¹⁶. Interestingly, judging from infection experiments using various scuticociliates plus identification outcome of 8 isolates acquired from olive flounders with symptom of ulcers and haemorrhages, *Miamiensis avidus* was suggested as the major aetiological agent of scuticociliatosis because of high pathogenicity and mortality rate compared with other scuticociliates^{14,17}.

Infection of bacteria could sustain serious damage to fish. Streptococcosis is known to be caused by a variety of streptococcal species; *Streptococcus parauberis*, *Streptococcus iniae*, *Streptococcus difficilis*, *Lactococcus garvieae*, *Lactococcus piscium*, *Vagococcus salmoninarum*, and *Carnobacterium piscicola*, and has become major nuisance in olive flounder farms¹⁸⁻²¹. In particular, *Streptococcus iniae*, *Lactococcus garvieae*, and *Streptococcus parauberis* have been introduced to be related with Streptococcosis in olive flounder¹⁹⁻²³.

The main issue of aquaculture industry is to reduce economic loss by preventing mortality of fish from various pathogens. A large number of immunologic studies have been proceeded about various immune-related genes against pathogen infection^{3,24-27}. A huge quantity of genomic information from next generation sequencing (NGS) technique has been gradually increasing for the last few years, indicating that researchers could approach more comprehensive understanding view about genome of organisms than when they research a single gene level. With development of wide-sized analysis methods, it is not difficult to figure out change of gene expression level after any chemical treatment or environmental change. Recently, studies to identify large-scale genes were conducted in the olive flounder genome for researches about vaccine, gonadal development, and sex determination²⁸⁻³⁰. In particular, characterizing of immune-related genes was reported in olive flounder spleen tissue³¹. A lot of studies reported earlier were focused on gene expression analysis of single pathogen and specifically defined the expression pattern of limited genes³²⁻³⁶. Further, infection by two or more pathogens were reported in the olive flounder genome^{37,38}. In order to solve these problem, we need plentiful genomic information to respond rapidly to multiple infection of pathogens. However, researches, which were comprehensively analysed about change of gene expression pattern by different type of pathogens, have not been reported in the olive flounder genome, so far.

In this research, we identified differentially expressed genes (DEGs) by transcriptome analysis and conducted gene ontology (GO) analysis with genes identified. Then, we tried to find important genes which showed consistently meaningful expression change in the results of three infection experiments. As a result, we determined 10 up-regulated genes and 57 down-regulated genes in common after infection of three pathogens. We aimed to provide essential genome information which is related with pathogen infection and explore the various consequences related to differential infections and find out the common strategies against specific candidates involved in disease progression in natural habitat of aquaculture.

Results

Statistical summary of transcriptome analysis. To profile gene expression after infection of three pathogens (VHSV, *Streptococcus parauberis*, and *Miamiensis avidus*), transcriptome analysis was conducted using gill tissues of olive flounders, respectively. We prepared twelve olive flounders (three un-infected individuals as control, three virus-infected, three bacteria-infected, and three parasite-infected individuals) to raise confidence. To gain the sufficient number of transcripts, twelve independent RNA samples acquired from normal and pathogen-infected olive flounder gill tissues were employed for construction of cDNA library. Then, these cDNA libraries were sequenced using Illumina HiSeq2500, generating the numbers of approximately 78.4 million, 65.2 million, and 45.7 million raw reads from three control samples, 44.0 million, 56.9 million, and 62.0 million raw reads from bacteria-infected samples, 41.2 million, 62.4 million, and 40.0 million raw reads from virus-infected samples, 39.6 million, 41.6 million, and 53.1 million raw reads from parasite-infected samples, respectively (Table 1). After trimming of low-quality reads and adaptor sequences, the number of clean reads acquired from control samples were average 62.3 million reads from control samples, average 53.5 million reads from bacteria-infected samples, average 48.0 million reads from virus-infected samples, and average 44.3 million reads from parasite-infected samples, respectively. Then, we checked gene coverage whether the reads that we acquired are sufficient for quantitative gene expression analysis (Supplementary Fig. 1). The clean reads were assembled into 120,880 transcript sequences acquired from transcriptome analysis, we identified total 40,100 genes involving novel 19,560 genes from transcript sequences using InterProScan database and non-redundant protein database in the NCBI (Table 2).

Identification of DEGs after viral infection. To figure out the effects of external pathogen for gene expression, we sorted out genes which showed expressional change after pathogens infection having *p*-value of <0.05 when compared with control sample. As shown in Table 3 and Fig. 1, the largest numbers of gene expression change were shown in VHSV infection group; total 12,415 DEGs were identified from transcriptome analysis. We showed information of DEGs derived from viral infection in Supplementary Table 1, as well as those after bacteria and parasite infection. In this result, we verified interesting phenomenon related in the number of DEGs after viral infection. Among 12,415 DEGs, total 11,051 DEGs were down-regulated after viral infection while

Description	Samples											
	U-I 1	U-I 2	U-I 3	B-I 1	B-I 2	B-I 3	V-I 1	V-I 2	V-I 3	P-I 1	P-I 2	P-I 3
<i>Reads</i>												
Number of raw reads	78,425,340	65,251,756	45,720,414	44,022,532	56,928,000	62,034,784	41,202,926	62,465,936	40,015,984	39,636,294	41,676,740	53,148,278
Number of clean reads	77,685,792	64,293,520	45,046,422	43,370,168	56,218,774	61,171,560	40,540,550	64,212,934	39,393,956	39,128,496	41,195,252	52,580,022
Number of mapped reads	35,293,624	34,011,030	21,768,778	23,241,737	25,467,484	26,640,040	25,215,623	37,509,022	19,159,186	6,587,353	13,259,405	11,685,297
Number of uniquely mapped reads	34,624,745	33,266,973	21,287,829	22,815,501	24,897,617	26,076,243	24,651,733	36,604,718	18,740,455	6,293,804	12,980,394	11,405,891
Mapping rate of unique reads	44.6%	51.7%	47.3%	52.6%	44.3%	42.6%	60.8%	59.8%	47.6%	16.1%	31.5%	21.7%

Table 1. Statistical summary of reads acquired from transcriptome analysis. U-I: uninfected, B-I: bacteria-infected, V-I: virus-infected, P-I: parasite-infected.

Description	Samples												Summary
	U-I 1	U-I 2	U-I 3	B-I 1	B-I 2	B-I 3	V-I 1	V-I 2	V-I 3	P-I 1	P-I 2	P-I 3	
<i>Gene</i>													
Known	1,707	1,720	1,504	1,641	1,617	1,669	1,670	1,751	1,664	1,348	1,588	1,243	1,969
Known (New Isoforms)	18,564	18,567	18,561	18,562	18,564	18,567	18,563	18,568	18,563	18,539	18,563	18,501	18,571
Novel	19,380	19,527	19,066	19,474	19,338	19,495	19,528	19,547	19,461	18,443	19,287	17,765	19,560
TOTAL	39,651	39,814	39,131	39,677	39,519	39,731	39,761	39,866	39,688	38,330	39,438	37,509	40,100
<i>Transcript</i>													
Known	25,572	25,078	24,574	24,900	25,079	24,966	24,479	24,618	24,189	23,061	24,516	22,945	27,216
Novel	93,402	93,533	93,017	93,505	93,345	93,513	93,532	93,527	93,416	92,260	93,297	91,281	93,664
TOTAL	118,974	118,611	117,591	118,405	118,424	118,479	118,011	118,145	117,605	115,321	117,813	114,226	120,880

Table 2. The number of genes and transcripts identified from transcriptome analysis. U-I: uninfected, B-I: bacteria-infected, V-I: virus-infected, P-I: parasite-infected.

1,364 DEGs showed up-regulation due to viral infection, indicating the number of down-regulated DEGs strikingly outnumbered than those of up-regulated DEGs as 8.1 folds after viral infection in olive flounder genome. 9,699 of 12,415 DEGs were annotated by InterProScan database and non-redundant protein database in the NCBI and used for functional analysis in next step.

To explore the functional enrichment of these DEGs, we performed GO enrichment analysis using DAVID tool³⁹ and sorted specially out the immune-related GO terms with p -value of <0.05 . As a result, 134 DEGs were enriched in 9 GO terms, including 'GO:0000045; autophagic vacuole assembly' ($p = 0.031353$), 'GO:0002376; immune system process' ($p = 0.024692$), 'GO:0006979; response to oxidative stress' ($p = 0.040376$), 'GO:0016032; viral process' ($p = 0.000325$), 'GO:0019221; cytokine-mediated signaling pathway' ($p = 0.016815$), 'GO:0030217; T cell differentiation' ($p = 0.032158$), 'GO:0033077; T cell differentiation in thymus' ($p = 0.024688$), 'GO:0042113; B cell activation' ($p = 0.037259$), and 'GO:0050853; B cell receptor signaling pathway' ($p = 0.020561$), in GO category 'biological process' (Supplementary Table 2).

Gene expression change pattern after bacterial infection. After analyzing DEGs and their functional prediction following viral infection, we identified the DEGs with p -value of <0.05 after infection with bacteria (*Streptococcus parauberis*) and parasite (*Miamiensis avidus*) (Table 3 and Fig. 1). First, infection of *Streptococcus parauberis* changed expression level of total 1,754 genes. Among them, 720 genes were upregulated, while 1,034 genes showed decreasing expression pattern. In case of bacterial infection, total number of DEGs after bacterial infection showed the number of one-seventh when compared with those of viral infection. Then, we carried out GO enrichment analysis with annotated 751 of 1,754 DEGs by genomic database and found that 11 DEGs were enriched in 5 immune-related GO terms {'GO:0001865; NK T cell differentiation' ($p = 0.034926$), 'GO:0002302; CD8-positive, alpha-beta T cell differentiation involved in immune response' ($p = 0.033205$), 'GO:0032609; Interferon-gamma production' ($p = 0.000022$), 'GO:0032633; Interleukin-4 production' ($p = 0.034926$), and 'GO:0042060; Wound healing' ($p = 0.012764$)} having p -value of <0.05 of 'biological process' GO category (Supplementary Table 2).

Gene expression change pattern after parasite infection. Last, we checked how infection of *Miamiensis avidus* affected gene expression pattern in olive flounder genome and selected genes which showed expression change (Supplementary Table 2). We identified 795 DEGs caused by infection of *Miamiensis avidus*;

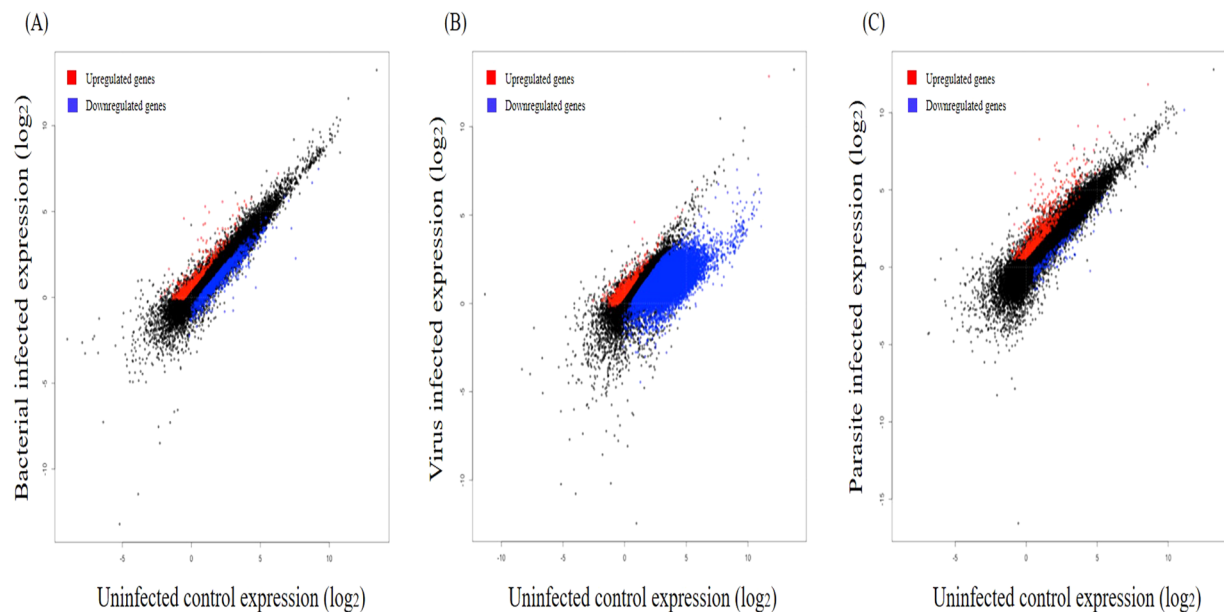


Figure 1. Global gene expression changes after infection by three pathogens. Genes expression in uninfected control samples plotted versus genes expression in bacterial infected samples (A), in virus infected samples (B), and in parasite infected samples (C). Genes changed after pathogen infection are colored in red and green for upregulated and downregulated, respectively ($p < 0.05$).

Pathogen	Expression change to control		
	Up	Down	Total
Virus	1,364	11,051	12,415
Bacteria	720	1,034	1,754
Parasite	640	155	795

Table 3. The number of DEGs after pathogens infection.

640 upregulated and 155 downregulated genes. By GO enrichment analysis with annotated 346 genes, we found only one immune-related GO term {GO:0050294; Steroid sulfotransferase activity, ($p = 0.011417$)} including one DEG.

Distributional pattern of total DEGs acquired from transcriptome analysis. After comparison of gene expression level among twelve transcriptome analysis, we identified DEGs after pathogen infection. Then, we focused on selection of genes showing expression change pattern after three types of pathogen infection in common. As a result, we summarized 10 up-regulated genes and 57 down-regulated genes, respectively (Fig. 2). We analyzed these 67 DEGs to identify their gene symbol correctly using their sequences in non-redundant protein database of the NCBI database, and 37 DEGs were annotated (Table 4). We showed the rest of 30 unannotated DEGs in Supplementary Table 3.

Discussion

With development of sequencing technique, numerous genomic researches have been reported to understand infection results by virus^{28,31,40}, bacteria^{26,41,42}, and parasite⁴³ in the olive flounder genome. A fundamental way to overcome disease outbreak from external pathogens is to approach from their genome level. It is essential to expand quantity of genomic information in pursuance of biological research about any target. Investigation of overall gene expression change after pathogen infection would provide clues of cause of biological damage. Gene regulation is essential for viruses, prokaryotes and eukaryotes as it increases the versatility and adaptability of an organism by allowing the cell to express protein when needed.

Phylogenetic diversity of pathogens (virus, bacteria and parasite) is also responsible for differential expression of genes in diseases. Each individual pathogen causes disease in a different way, which makes it challenging to understand the basic biology of infection. In this study, we understood the relation between three types of pathogen infection and differential gene expression in the olive flounder genome through transcriptome analysis, respectively. The diverse pathogens used in this study, carry specific antigenic variations, which refers to the specific mechanism by which an infectious agent infect the fish and progress the disease. Transcriptome analysis help us to understand the progression of disease in fish through pathogen infection based on diversity of pathogen (virus, bacteria and parasite). This study shows differentially expressed genes were up- and down-regulated

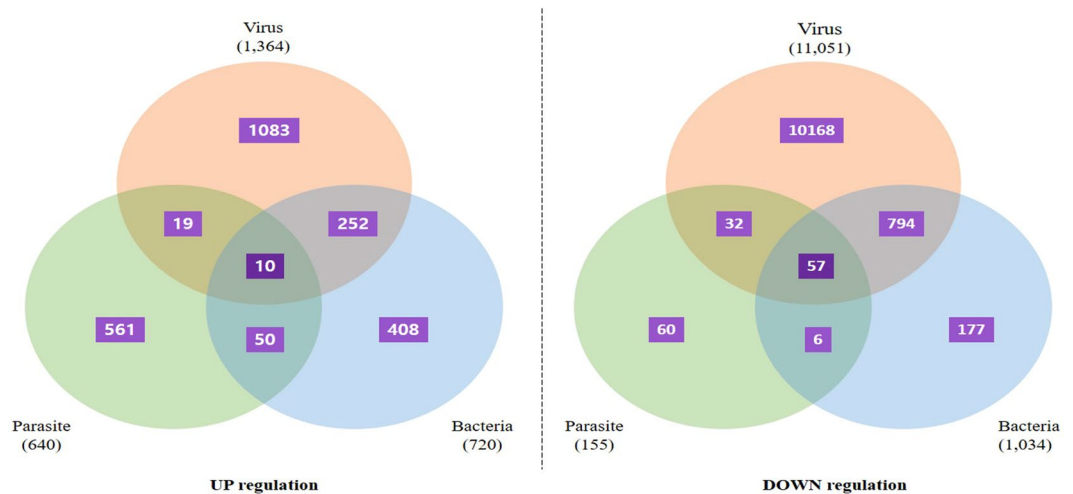


Figure 2. Distribution of DEGs in three pathogens group (bacteria, virus, and parasite). Venn diagram show the number of DEGs among bacteria, virus, and parasite. 10 up-regulated and 57 down-regulated DEGs were overlapped in three pathogens group on common. The up-regulation of 1083 genes (virus), 408 genes (bacteria), and 561 genes (parasites). These genes were specific for each pathogen so can be used as candidate genes for vaccination or therapeutic agents. In case of the down-regulation of 10168 gene (virus), 177 gene (bacteria) and 60 gene (parasites) in infection of fish, these genes were specific for specific pathogens so can be used as a diagnosis marker for specific pathogens.

at different extend in fish tissue. Interestingly, virus and bacteria have more down-regulated genes while parasite have more up-regulated genes. This data signifies the fact that fish immune system interacts with bacteria and virus with the same strategy, while with parasite different due to difference in mode of infections between them.

For efficient prevention against pathogen, it is important to understand which genes were activated/repressed after pathogen infection because their expression change means variation of metabolic system in body. In this study, we identified total number of 12,415 in VHSV infection group, 1,754 in *Streptococcus parauberis* infection group, and 795 DEGs from *Miamiensis avidus* infection group, respectively (Table 3). Given the difference in the number of DEGs among three pathogen groups, these results seemed that virus had the most impact on gene expression mechanism in the olive flounder genome among three pathogens. Interestingly, 11,051 DEGs (89% of all DEGs) showed down-regulated pattern after viral infection. This phenomenon that global gene expression was decreased by viral infection must cause pathogenic disease by affecting immune-related gene expression level, finally leads to death. This view was supported by our findings (Supplementary Table 2), which showed expression decrease pattern of all genes in the immune-related GO terms. Especially, GO terms in viral infection group showed that all genes tend to be down-regulated after pathogen infection, indicating loss of resistance against pathogens by down-regulating the expression of immune-related genes. Like this situation, functional information of genes acquired from GO enrichment could help researcher to figure out critical biological pathway against any external factor.

The immune mechanism in fishes is composed of a set of cellular and humoral system and divided into innate (inherit), and adaptive (acquired) substances. The understanding of fish immune system structure and function is essential for the development of new technologies and products to improve productivity. The transcriptome analysis bring exposure to basic difference in expression profile of all pathogens in the host. These differences were due to nature of parasite, their mode of infection, antigenic variations and many other factors. Additionally, along with all above differences, disease progressed in host due to external surface variation of pathogens (viral, parasite and bacterial) and their appropriate recognition by host immune systems for making the basis to initiate microbial clearance^{44,45}. Disease research requires the knowledge of important key factors like method of avoiding host immune surveillance, antigenic variations, subversion of immune responses through phagocyte and inhibition of cytokines and chemokines in common with pathogen infections (viral, bacterial and parasite)^{44,45}. On the other hand, the disease progression was different in accordance to type of pathogens. In case of viral infection, understanding of complement inhibition and blockade of cellular immunity is the most important, while parasite and bacterial infections required knowledge and research of innate pathway and acquired immunity^{44,45}. Our analysis indicates the complexity and difference of expression profile could be due to all the above reasons. In addition, important basis of fish vaccine is depending on innate and adaptive immunity⁴⁶. There were many vaccine types which depend upon antigens, live microorganisms or specific DNA segment of pathogens or polyvalent vaccines. All above vaccines required complete knowledge of pathogenicity and deep research of efficacy⁴⁷. Our study clearly indicates about various immune and antigenic genes which can be chosen for pathways analysis and use for therapeutic agents or as some vaccine candidates (Supplementary Table 2).

Despite of their different infection pathway, we wondered common DEGs which were affected from infection. As shown in Fig. 2, total 67 DEGs (10 up-regulation and 57 down-regulation) were identified in common in three pathogens, and 1 of 10 up-regulated and 36 of 57 DEGs were annotated by genomic database (Table 4;

Gene Expression Change (Control → Infection)	Gene Accession	Description	Fold change (log2) (Bacteria/control)	Fold change (log2) (Virus/control)	Fold change (log2) (Parasite/control)	Predicted gene symbol
UP	GENE19852.r1	Fibroblast growth factor 4 [Source:SWISS;Acc:P48804]	0.658	0.661	0.782	FGF4
Down	GENE00707.r1	Acidic mammalian chitinase [Source:SWISS;Acc:Q91XA9]	-0.540	-1.386	-1.367	Chia
	GENE16184.r1	Rab GDP dissociation inhibitor beta [Source:SWISS;Acc:P50395]	-1.739	-1.399	-1.218	GDI2
	GENE11810.r1	Neuronal acetylcholine receptor subunit alpha-7 [Source:SWISS;Acc:P22770]	-1.041	-1.566	-2.009	CHRNA7
	GENE04169.r1	Histidine decarboxylase [Source:SWISS;Acc:P23738]	-1.177	-1.173	-1.266	Hdc
	GENE11460	Semaphorin-3E [Source:SWISS;Acc:O42237]	-1.454	-2.466	-1.203	SEMA3E
	GENE21840.r1	Cysteine-rich secretory protein LCCL domain-containing 1 [Source:SWISS;Acc:Q8CGD2]	-0.705	-1.769	-1.146	Crispld1
	GENE23270.r1	Cx9C motif-containing protein 4 [Source:SWISS;Acc:P56277]	-1.562	-3.848	-2.557	CMC4*
	GENE06025.r1	Protein phosphatase 1 regulatory subunit 3C-B [Source:SWISS;Acc:Q6P950]	-0.829	-1.627	-1.308	ppp1r3cb
	GENE18715.r1	Collagen alpha-2(I) chain [Source:SWISS;Acc:O46392]	-3.069	-5.000	-2.317	COL1A2*
	GENE18863	2-aminoethanethiol dioxygenase [Source:SWISS;Acc:Q6PDY2]	-1.029	-2.165	-1.399	Ado
	GENE05041.r1	AP-1 complex subunit sigma-3 [Source:SWISS;Acc:Q7TN05]	-1.146	-1.544	-1.340	Ap1s3
	GENE03465	Homeobox protein Nkx-3.1 [Source:SWISS;Acc:Q99801]	-1.078	-3.351	-1.063	NKX3-1
	GENE24080.r1	Transmembrane protein 205 [Source:SWISS;Acc:A1L2F6]	-0.743	-2.695	-0.980	tmem205
	GENE14826.r1	Ethanolamine-phosphate phospho-lyase [Source:SWISS;Acc:Q7SY54]	-1.289	-1.961	-1.274	etnpl
	GENE01095.r1	Alcohol dehydrogenase 1 [Source:SWISS;Acc:P26325]	-0.750	-1.348	-0.906	ADH1_GADMC
	GENE12085.r1	Pyridine nucleotide-disulfide oxidoreductase domain-containing protein 2 [Source:SWISS;Acc:Q68FT3]	-0.953	-1.658	-0.839	Pyroxd2
	GENE22874.r1	Potassium voltage-gated channel subfamily KQT member 3 [Source:SWISS;Acc:Q8K3F6]	-0.616	-0.475	-0.785	Kcnq3
	GENE06563	Protein Hook homolog 3 [Source:SWISS;Acc:Q6GQ73]	-1.723	-4.109	-1.638	hook3
	GENE01318.r1	Mitochondrial peptide methionine sulfoxide reductase [Source:SWISS;Acc:P54149]	-0.693	-1.130	-0.953	MSRA
	GENE02237.r1	Anterior gradient protein 3 homolog [Source:SWISS;Acc:Q8TD06]	-1.288	-3.663	-1.523	AGR3
	GENE11050.r1	Viral T-cell receptor beta chain-like T17T-22 [Source:SWISS;Acc:P11364]	-1.211	-3.670	-1.053	V-TCR
	GENE09774	Claudin-12 [Source:SWISS;Acc:Q5R9K1]	-0.685	-2.185	-0.839	CLDN12
	GENE11823.r1	Proline-rich protein 5-like [Source:SWISS;Acc:A2AVJ5]	-0.924	-1.909	-0.949	Prr5l
	GENE03568.r1	Urea transporter 2 [Source:SWISS;Acc:Q28614]	-2.107	-4.956	-2.405	SLC14A2*
	GENE06547	Krueppel-like factor 5 [Source:SWISS;Acc:Q9Z0Z7]	-1.264	-2.379	-1.251	Klf5
	GENE09838.r1	Integrin alpha-10 [Source:SWISS;Acc:O75578]	-1.017	-2.154	-1.004	ITGA10
	GENE21626.r1	Hydroxycarboxylic acid receptor 2 [Source:SWISS;Acc:Q8TDS4]	-1.033	-2.299	-1.053	HCAR2
	GENE01511.r1	Ectonucleoside triphosphate diphosphohydrolase 5 [Source:SWISS;Acc:O75356]	-1.063	-1.592	-0.818	ENTPD5
	GENE15828.r1	Matrix metalloproteinase-20 [Source:SWISS;Acc:O18767]	-1.145	-2.128	-0.738	MMP20
	GENE06887.r1	Osteocalcin [Source:SWISS;Acc:P02823]	-2.740	-3.546	-1.659	bglap*
	GENE01596.r1	GTP cyclohydrolase 1 feedback regulatory protein [Source:SWISS;Acc:Q6PBT6]	-1.253	-2.255	-0.904	gchfr
	GENE16851.r1	Aminopeptidase N [ECO:0000312][EMBL:ACZ95799.1] [Source:SWISS;Acc:O57579]	-1.504	-3.185	-2.428	ANPEP*
XLOC_002910	Trypsin-1 [Source:SWISS;Acc:P35031]	-1.401	-1.350	-2.147	TRY1_SALSA	
XLOC_021180	Collagen alpha-1(I) chain [Source:SWISS;Acc:P02457]	-2.092	-5.204	-2.025	COL1A1*	
XLOC_036089	AT-hook-containing transcription factor [Source:SWISS;Acc:Q80VW7]	-1.577	-2.290	-1.507	Akna	
XLOC_029355	Collagen alpha-3(VI) chain [Source:SWISS;Acc:P15989]	-1.301	-3.553	-0.819	COL6A3	

Table 4. List of annotated DEGs among common gene after three pathogens infection. *Six genes with asterisk are representative genes which have fold change (log2) of -2.0 in at least two pathogen groups. $p < 0.05</math>.$

Supplementary Table 3). These common genes can be used for diagnosis purpose as a candidate gene for any kind of pathogens (virus, bacteria and parasites). Here, we mentioned that representative six genes (ANPEP; Alanyl Aminopeptidase, Membrane, BGLAP; Bone gamma-carboxyglutamic acid-containing protein, CMC4;

C-X9-C Motif Containing 4, COL1A1; Collagen Type I Alpha 1 Chain, COL1A2; Collagen Type I Alpha 2 Chain, SLC14A2; Solute Carrier Family 14 Member 2) which showed highest expression change (down regulation) with fold change (\log_2) of < -2.0 on at least two infection groups. In this study, we investigated all the above candidate genes and found their role in disease progression. We have listed these genes and their role in below headings.

ANPEP, called as Gene Aminopeptidase N (APN), is metallopeptidase that exerts strong influence on various immune response mechanisms. For example, APN has been known to cause decomposition of cytokines and peptides used by neurons^{48–50}, and acts as receptor for viruses^{51,52}. In addition, relation between expression level of APN and stimulated T-cell was reported⁵³. Recently, it was suggested that APN controlled the balance of innate immune and adaptive immune by regulating TLR4 signal transduction pathway in myeloid cells⁵⁴.

BGLAP, also known as Osteocalcin, is a noncollagenous protein, mainly found in bone, which needs vitamin K for its synthesis. This protein was thought to play a role in calcium ion homeostasis and used as biological marker for bone formation⁵⁵. In addition, it concerns in endocrine regulation, especially in digestive system, by stimulating release of insulin hormone from β -cell of the pancreas and adiponectin hormone from fat cells, respectively⁵⁶. As well as these function, it has been reported to take a role in promotion of energy availability and sexual maturation of male by stimulation of testosterone biosynthesis^{57,58}.

CMC4, called as MTCP1, has been mainly reported to be related in various diseases. It was reported that MTCP1 gene affected T-cell homeostasis prior to process of leukemogenesis in transgenic mice⁵⁹. Although the function of this gene has been entirely discovered, regulation error of MTCP1 gene affected on cell survival and cell growth⁶⁰. Besides, this gene was known to be related in the pathogenesis of a subset of T-cell lymphoproliferative diseases^{61,62}.

COL1A1 and COL1A2 encodes the pro- α 1 chain and the pro- α 2 chain protein, respectively. The type I collagen, which is comprised by two pro- α 1 chains and one pro- α 2 chain, plays a role in reinforcement and support in most of all connective tissues such as bone, cartilage, skin, and tendon, and offers those tissues rigidity and elasticity^{63,64}. This protein was reported to stimulate expression of pro-inflammatory cytokines and professional phagocytes in teleost fish gilthead seabream^{65,66}. In addition, it has been reported that receptor-mediated interaction which is formed in between cells and collagen molecule might affect in wound healing, inflammatory, and immune response by activating various factors such as cytokines, growth factor, and matrix metalloprotease^{63,66–70}.

SLC14A2, also is known Urea transporter 2 (HUT2), is important gene involved in urea transport and play role in physiology. In mammals, two types of urea transporter (SLC14A1 and SLC14A2) has been reported⁷¹ and were regulated by vasopressin hormone⁷². The kidney uses urea to maintain the appropriate concentration and volume of blood. Without control of these proteins, organism would result in extreme damage in urinary system. Besides, a previous study has reported that genetic variation including nucleotide change is known to significantly influence blood pressure (BP) and metabolism syndrome^{73,74}.

As shown in Supplementary Table 2, immune-related DEGs were revealed as results of three pathogens infection. Infection of pathogens caused activation of immune system to respond to invasion of harmful external elements, indicated that change of expression level of immune-related genes. The down-regulation of gene could sequentially influence on expression of various molecules positioned in down-streams in metabolic pathway. In this view, CD13, which is one of down-regulated genes by infection of three pathogens on common, was reported to inactivate interleukin 8⁴⁹. Representative function of this cytokine is to induce migration of neutrophils and granulocytes toward infection site. In addition, absence of CD13 considerably improves cross-presentation of soluble antigen via regulation of receptor-mediated uptake⁷⁵. Thus, decrease of CD13 expression consequentially might activate immune response in the olive flounder. MTCP1 gene induced malignant T-cell transformation⁵⁹ and was related in the leukemogenic process of mature T-cell proliferation⁶¹. This gene was thought to maintain balance of immune response by T-cell. The innate immune system mediates the initial inflammatory response by pathogen infection or injury. For rapid response against external pathogens, infected cells secrete various cytokines to induce effector cells and complements. The type I collagen, which is comprised by proteins coded from COL1A1 and COL1A2, was involved in the expression of pro-inflammatory cytokines in the innate immune system. However, two genes (COL1A1 and COL1A2) showed decreasing expression pattern after infection in our study. Given sampling period (7 days from infection) of olive flounders for this study, it might be explained that the adaptive immune response was activated in the olive flounder genome. It is hard to understand comprehensively about immune system of fish genome. However, our results were expected to contribute for further study by extend of genomic knowledge in the olive flounder.

In conclusion, this study is helpful in understanding infection of the diversified pathogens (antigenic variation) and their role in disease progression in the olive flounder. The differentially expressed genes identified from transcriptome analysis using three types of pathogens could be useful to study the basic diagnosis and therapeutic mechanisms, and offer opportunities for designing the appropriate vaccines or drug targets for pathogen specific candidate genes. Because of lack of genomic information or using one external infection factor, previous studies have been limited to understand global expression pattern of whole genes in the olive flounder genome. We hope that this research would contribute to achieve great outcome in various biological field.

Materials and Methods

Ethical statement. All experiments with the olive flounders in this study were carried out in accordance with the guidelines and regulation approved by Ethical Committee of Pukyong National University.

Preparation of olive flounder gill tissues. Gill tissues from twelve olive flounder (BW = ~50 g, n = 3/group) including healthy and infected fish with each pathogen were used for this study. Briefly, healthy fish (non-challenged), sampled fish at 7 days post challenge (dpc) with *S. parauberis* at 5.06×10^3 CFU/fish in 1/3 seawater of 21 °C, sampled fish at 7 dpc with VHSV at 106 PFU/fish in 1/3 seawater of 19 °C, and sampled fish

at 26 days post exposure to *M. avidus* at 3×10^3 cells/ml in 1/3 seawater of 20 °C, respectively, were used in this study. We used total twelve samples including three samples per each group (uninfected, virus-infected, bacteria-infected, and parasite-infected) to minimize difference of genomic feature shown from individuals and understand common pattern of gene expression change in each group.

RNA preparation for NGS. The samples were stored in RNAlater RNA Stabilization (Qiagen, Germany) solution. RNeasy Mini Kit columns were used to extract total RNA according to the manufacturer's protocol (Qiagen). In order to measure RNA quality, an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands) was used. Integrity values to RNA measurement were confirmed by the RNA integrity number (RIN). Total RNA quantity was assessed with Infinite 200 PRO NanoQuant Spectrophotometer (TECAN, Switzerland).

Construction of cDNA libraries for transcriptome analysis. Building of transcriptome libraries were conducted by Illumina's TruSeq RNA protocol, and 1–2 µg of total RNA were used in each samples. AMPure XP beads (BECKMAN COULTER) and Ambion Fragmentation Reagents kit (Ambion, Austin, TX) were used for extraction of Poly(A)+ RNA and their fragment, respectively. As the following steps, cDNA synthesis, end-repair, A-base addition, and ligation of the Illumina indexed adapters were carried out according to Illumina's protocol. The size-selected 250–300 bp cDNA fragments were loaded on a 3% Nusieve 3:1 (Lonza) agarose gel for libraries. The cDNA fragments were recovered using QIAEX II gel extraction reagents (Qiagen), and amplified using Phusion DNA polymerase (New England Biolabs) for 14 PCR cycles. The amplified libraries were purified by AMPure XP beads, their concentration and product sizes were assessed on an Agilent 2100 Bioanalyzer. Sequencing of paired-end libraries were conducted with the Illumina HiSeq2500, (2×100 nucleotide read length).

Transcriptome analysis and differential gene expression. Transcriptome analysis were carried out with the RNAseq Tuxedo protocol. Mapping of Sequences were conducted against the Olive flounder draft genome (Submitted at present) using TopHat v2.0.9 with default options for paired-end sequences. Transcript expression were estimated using the Cufflinks program v2.1.1. Total sequencing reads were subjected to preprocessing as follows: adapter trimming was performed using cutadapt with default parameters, and quality trimming (Q30) was performed using FastQC with default parameters. Processed reads were mapped to the Olive flounder draft genome (Submitted at present) using tophat and cufflink with default parameters⁷⁶. The differential analysis was performed using Cuffdiff⁷⁶ using default parameters. Further, the FPKM values from Cuffdiff were normalized and quantitated using R Package Tag Count Comparison (TCC)⁷⁷ to determine statistical significance (e.g., P values) and differential expression (e.g., fold changes.). Through these statistics analysis, we sorted DEGs having $p < 0.05$ and showed them as results.

Gene ontology analysis. DEG set for GO analysis was acquired from transcriptome analysis. DEGs were annotated from InterProScan database and non-redundant protein database in the NCBI. DAVID and uniprot tool were used for exploring the functional enrichment of these DEGs and sorting specially out the immune-related GO terms with p -value of < 0.05 , respectively.

References

- Aoki, T. & Hirono, I. Immune relevant genes of Japanese flounder, *Paralichthys olivaceus*. *Comparative biochemistry and physiology. Part D, Genomics & proteomics* **1**, 115–121, <https://doi.org/10.1016/j.cbd.2005.07.002> (2006).
- Takano, T., Iwahori, A., Hirono, I. & Aoki, T. Development of a DNA vaccine against hirame rhabdovirus and analysis of the expression of immune-related genes after vaccination. *Fish & shellfish immunology* **17**, 367–374, <https://doi.org/10.1016/j.fsi.2004.04.012> (2004).
- Liu, C. S., Sun, Y., Hu, Y. H. & Sun, L. Identification and analysis of the immune effects of CpG motifs that protect Japanese flounder (*Paralichthys olivaceus*) against bacterial infection. *Fish & shellfish immunology* **29**, 279–285, <https://doi.org/10.1016/j.fsi.2010.04.012> (2010).
- Schütze, H., Mundt, E. & Mettenleiter, T. C. Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. *Virus Genes* **19**, 59–65 <https://link.springer.com/article/https://doi.org/10.1023/A:1008140707132> (1999).
- Skall, H. F., Olesen, N. J. & Møllergaard, S. Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming - a review. *Journal of fish diseases* **28**, 509–559, <https://doi.org/10.1111/j.1365-2761.2005.00654.x> (2005).
- Takano, R., Nishizawa, T., Aritmoto, M. & Muroga, K. Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild Japanese flounder, *Paralichthys olivaceus*. *Bulletin of the European Association of Fish Pathologists* **20**, 186–192, http://eafp.org/download/2000-Volume20/Issue%205/20_186.pdf (2000).
- Kim, S. M., Lee, J. I., Hong, M. J., Park, H. S. & Park, S. I. Genetic relationship of the VHSV (viral hemorrhagic septicemia virus) isolated from cultured olive flounder, *Paralichthys olivaceus* in Korea. *Journal of fish pathology* **16**, 1–12, http://www.koreascience.or.kr/article/ArticleFullRecord.jsp?cn=HGOPB8_2003_v16n1_1 (2003).
- Kim, W. S. *et al.* An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed olive flounder *Paralichthys olivaceus* in Korea. *Aquaculture* **296**, 165–168, <https://doi.org/10.1016/j.aquaculture.2009.07.019> (2009).
- Isshiki, T., Nishizawa, T., Kobayashi, T., Nagano, T. & Miyazaki, T. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. *Diseases of aquatic organisms* **47**, 87–99, <https://doi.org/10.3354/dao047087> (2001).
- Iglesias, R. *et al.* *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida) as the causative agent of scuticociliatosis in farmed turbot *Scophthalmus maximus* in Galicia (NW Spain). *Diseases of aquatic organisms* **46**(47–55), 2001, <https://doi.org/10.3354/dao046047> (2001).
- Imai, S., Tsurimaki, S., Goto, E., Wakita, K. & Hatai, K. Tetrahymena infection in guppy, *Poecilia reticulata*. *Fish pathology* **35**, 67–72, https://www.jstage.jst.go.jp/article/jsfp1966/35/2/35_2_67/_pdf (2000).
- Munday, B. L., O'Donoghue, P. J., Watts, M., Rough, K. & Hawkesford, T. Fatal encephalitis due to the scuticociliata *Uronema nigricans* in sea-caged, southern bluefin tuna *Thunnus maccoyii*. *Diseases of aquatic organisms* **30**, 17–25, <https://doi.org/10.3354/dao030017> (1997).

13. Jee, B. Y., Kim, Y. C. & Park, M. S. Morphology and biology of parasite responsible for scuticociliatosis of cultured olive flounder *Paralichthys olivaceus*. *Diseases of aquatic organisms* **47**, 49–55, <https://doi.org/10.3354/dao047049> (2001).
14. Jung, S. J., Kitamura, S., Song, J. Y., Jung, I. Y. & Oh, M. J. Complete small subunit rRNA gene sequence of the scuticociliate *Miamiensis avidus* pathogenic to olive flounder *Paralichthys olivaceus*. *Diseases of aquatic organisms* **64**, 159–162, <https://doi.org/10.3354/dao064159> (2005).
15. Kim, S. M. *et al.* Pseudocohnilembus persalinus (Ciliophora: Scuticociliatida) is an additional species causing scuticociliatosis in olive flounder *Paralichthys olivaceus*. *Dis. Aquat. Org.* **62**, 239–244, <https://doi.org/10.3354/dao062239> (2004a).
16. Kim, S. M., Cho, J. B., Kim, S. K., Nam, Y. K. & Kim, K. H. Occurrence of scuticociliatosis in olive flounder *Paralichthys olivaceus* by *Phiasterides dicentrarchi* (Ciliophora: Scuticociliatida). *Diseases of aquatic organisms* **62**, 233–238, <https://doi.org/10.3354/dao062233> (2004b).
17. Song, J. Y. *et al.* Pathogenicity of Miamiensis avidus (syn. Philasterides dicentrarchi), Pseudocohnilembus persalinus, Pseudocohnilembus hargisi and Uronema marinum (Ciliophora, Scuticociliatida). *Diseases of aquatic organisms* **83**, 133–143, <https://doi.org/10.3354/dao02017> (2009).
18. Baek, G. W., Kim, J. H., Gomez, D. K. & Park, S. C. Isolation and characterization of *Streptococcus* sp. from diseased flounder (*Paralichthys olivaceus*) in Jeju Island. *Journal of veterinary science* **7**, 53–58, <https://doi.org/10.4142/jvs.2006.7.1.53> (2006).
19. Shin, G. W. *et al.* Discrimination of streptococcosis agents in olive flounder (*Paralichthys olivaceus*). *Bulletin of the European Association of Fish Pathologists* **26**, 68–79 https://www.researchgate.net/publication/228490172_Discrimination_of_streptococcosis_agents_in_olive_flounder_Paralichthys_olivaceus (2006).
20. Kusuda, R. & Kawai, K. Bacterial Diseases of Cultured Marine Fish in Japan. *Fish pathology* **33**, 221–227, https://www.jstage.jst.go.jp/article/jfsfp1966/33/4/33_4_221/_pdf (1998).
21. Mata, A. I. *et al.* Multiplex PCR assay for detection of bacterial pathogens associated with warm-water Streptococcosis in fish. *Applied and environmental microbiology* **70**, 3183–3187, <https://doi.org/10.1128/AEM.70.5.3183-3187.2004> (2004).
22. Domènech, A. *et al.* Streptococcosis in cultured turbot (*Schophthalmus maximus*) associated with *Streptococcus parauberis*. *Journal of fish diseases* **19**, 33–38, <https://doi.org/10.1111/j.1365-2761.1996.tb00117.x> (1996).
23. Nho, S. W. *et al.* Phenotypic characteristics of *Streptococcus iniae* and *Streptococcus parauberis* isolated from olive flounder (*Paralichthys olivaceus*). *FEMS microbiology letters* **293**, 20–27, <https://doi.org/10.1111/j.1574-6968.2009.01491.x> (2009).
24. Hwang, S. D., Asahi, T., Kondo, H., Hirono, I. & Aoki, T. Molecular cloning and expression study on Toll-like receptor 5 paralogs in Japanese flounder *Paralichthys olivaceus*. *Fish & shellfish immunology* **29**, 630–638, <https://doi.org/10.1016/j.fsi.2010.06.011> (2010).
25. Wang, L. *et al.* Characterization and functional analysis of a novel C1q-domain-containing protein in Japanese flounder (*Paralichthys olivaceus*). *Developmental and comparative immunology* **67**, 322–332, <https://doi.org/10.1016/j.dci.2016.09.001> (2017).
26. Cha, I. S. *et al.* Cathepsins in the kidney of olive flounder, *Paralichthys olivaceus*, and their responses to bacterial infection. *Developmental and comparative immunology* **38**, 538–544, <https://doi.org/10.1016/j.dci.2012.08.005> (2012).
27. Kim, Y. O. *et al.* Molecular cloning and expression analysis of two hepcidin genes from olive flounder *Paralichthys olivaceus*. *Bioscience, biotechnology, and biochemistry* **69**, 1411–1414, <https://doi.org/10.1271/bbb.69.1411> (2005).
28. Hwang, J. Y. *et al.* RNA-Seq transcriptome analysis of the olive flounder (*Paralichthys olivaceus*) kidney response to vaccination with heat-inactivated viral hemorrhagic septicemia virus. *Fish & shellfish immunology* **62**, 221–226, <https://doi.org/10.1016/j.fsi.2017.01.016> (2017).
29. Zhang, W. *et al.* Transcriptome analysis of the gonads of olive flounder (*Paralichthys olivaceus*). *Fish physiology and biochemistry* **42**, 1581–1594, <https://doi.org/10.1007/s10695-016-0242-2> (2016).
30. Fan, Z. *et al.* Gonadal transcriptome analysis of male and female olive flounder (*Paralichthys olivaceus*). *BioMed research international* **2014**, 291067, <https://doi.org/10.1155/2014/291067> (2014).
31. Huang, L. *et al.* De Novo assembly of the Japanese flounder (*Paralichthys olivaceus*) spleen transcriptome to identify putative genes involved in immunity. *PLoS One* **10**, e0117642, <https://doi.org/10.1371/journal.pone.0117642> (2015).
32. Hwang, J. Y. *et al.* Differentially expressed genes after viral haemorrhagic septicaemia virus infection in olive flounder (*Paralichthys olivaceus*). *Veterinary microbiology* **193**, 72–82, <https://doi.org/10.1016/j.vetmic.2016.05.024> (2016).
33. Kim, J. U. & Jung, S. J. cDNA microarray analysis of viral hemorrhagic septicemia infected olive flounder, *Paralichthys olivaceus*: immune gene expression at different water temperature. *Journal of fish pathology* **27**, 1–9, <https://doi.org/10.7847/jfp.2014.27.1.001> (2014).
34. Lee, Y. M. *et al.* Transcriptional analysis of olive flounder lectins in response to VHSV infection. *Journal of fish pathology* **29**, 13–23, <https://doi.org/10.7847/jfp.2016.29.1.013> (2016).
35. Jang, Y. H., Subramanian, D., Won, S. H. & Heo, M. S. Immune response of olive flounder (*Paralichthys olivaceus*) infected with the myxosporean parasite *Kudoa septempunctata*. *Fish & shellfish immunology* **67**, 172–178, <https://doi.org/10.1016/j.fsi.2017.06.019> (2017).
36. Park, E. M. *et al.* Cloning and Expression Analysis of Cathepsin D in the Olive Flounder *Paralichthys olivaceus*. *Bioscience, Biotechnology, and Biochemistry* **73**, 1856–1859, <https://doi.org/10.1271/bbb.80822> (2009).
37. Jung, S. J. *et al.* Distribution of marine birnavirus in cultured olive flounder *Paralichthys olivaceus* in Korea. *The journal of microbiology* **46**, 265–273, <https://doi.org/10.1007/s12275-008-0004-7> (2008).
38. Kotob, M. H., Menanteau-Ledouble, S., Kumar, G., Abdelzaher, M. & El-Matbouli, M. The impact of co-infections on fish: a review. *Veterinary research* **48**, 26, <https://doi.org/10.1186/s13567-016-0383-4> (2016).
39. Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* **4**, 44–57, <https://doi.org/10.1038/nprot.2008.211> (2009).
40. Zhang, B. C., Zhang, J. & Sun, L. In-depth profiling and analysis of host and viral microRNAs in Japanese flounder (*Paralichthys olivaceus*) infected with megalocytivirus reveal involvement of microRNAs in host-virus interaction in teleost fish. *BMC Genomics* **15**, 878, <https://doi.org/10.1186/1471-2164-15-878> (2014).
41. Moon, J. Y. *et al.* A cDNA microarray analysis to identify genes involved in the acute-phase response pathway of the olive flounder after infection with *Edwardsiella tarda*. *Veterinary immunology and immunopathology* **161**, 49–56, <https://doi.org/10.1016/j.vetimm.2014.07.002> (2014).
42. Cha, I. S. *et al.* Heat shock protein profiles on the protein and gene expression levels in olive flounder kidney infected with *Streptococcus parauberis*. *Fish & shellfish immunology* **34**, 1455–1462, <https://doi.org/10.1016/j.fsi.2013.03.355> (2013).
43. Kang, J., Park, C., Jang, Y., Ahn, M. & Shin, T. Lectin histochemistry of *Kudoa septempunctata* genotype ST3-infected muscle of olive flounder (*Paralichthys olivaceus*). *Parasite* **23**, 21, <https://doi.org/10.1051/parasite/2016021> (2016).
44. Finlay, B. B. & McFadden, G. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. *Cell* **124**, 767–782, <https://doi.org/10.1016/j.cell.2006.01.034> (2006).
45. Alvarez-Pellitero, P. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Veterinary immunology and immunopathology* **126**, 171–198, <https://doi.org/10.1016/j.vetimm.2008.07.013> (2008).
46. Zhu, L. Y., Nie, L., Zhu, G., Xiang, L. X. & Shao, J. Z. Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. *Developmental and comparative immunology* **39**, 39–62, <https://doi.org/10.1016/j.dci.2012.04.001> (2013).
47. Muktar, Y., Tesfaye, S. & Tesfaye, B. Present Status and Future Prospects of Fish Vaccination: A Review. *Journal of veterinary science & technology* **7**, 2, <https://doi.org/10.4172/2157-7579.1000299> (2016).

48. Furuhashi, M. *et al.* *In vitro* degradation of opioid peptides by human placental aminopeptidase M. *Experimental and clinical endocrinology* **92**, 235–237, <https://doi.org/10.1055/s-0029-1210807> (1988).
49. Kanayama, N. *et al.* Inactivation of interleukin-8 by aminopeptidase N (CD13). *Journal of leukocyte biology* **57**, 129–134, <http://www.jleukbio.org/content/57/1/129.long> (1995).
50. Larsen, S. L., Pedersen, L. O., Buus, S. & Stryhn, A. T cell responses affected by aminopeptidase N (CD13)-mediated trimming of major histocompatibility complex class II-bound peptides. *The Journal of experimental medicine* **184**, 183–189, <https://doi.org/10.1084/jem.184.1.183> (1996).
51. Yeager, C. L. *et al.* Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* **357**, 420–422, <https://doi.org/10.1038/357420a0> (1992).
52. Söderberg, C. *et al.* CD13 (human aminopeptidase N) mediates human cytomegalovirus infection. *Journal of virology* **67**, 6576–6585, <http://jvi.asm.org/content/67/11/6576.long> (1993).
53. Wex, T. *et al.* The activation-dependent induction of APN-(CD13) in T-cells is controlled at different levels of gene expression. *FEBS letters* **412**, 53–56, [https://doi.org/10.1016/S0014-5793\(97\)00738-2](https://doi.org/10.1016/S0014-5793(97)00738-2) (1997).
54. Ghosh, M., Subramani, J., Rahman, M. M. & Shapiro, L. H. CD13 restricts TLR4 endocytic signal transduction in inflammation. *The Journal of immunology* **194**, 4466–4476, <https://doi.org/10.4049/jimmunol.1403133> (2015).
55. Bharadwaj, S., Naidu, A. G., Betageri, G. V., Prasadarao, N. V. & Naidu, A. S. Milk ribonuclease-enriched lactoferrin induces positive effects on bone turnover markers in postmenopausal women. *Osteoporosis international* **20**, 1603–1611, <https://doi.org/10.1007/s00198-009-0839-8> (2009).
56. Lee, N. K. *et al.* Endocrine regulation of energy metabolism by the skeleton. *Cell* **130**, 456–469, <https://doi.org/10.1016/j.cell.2007.05.047> (2007).
57. Karsenty, G. & Oury, F. Regulation of male fertility by the bone-derived hormone osteocalcin. *Molecular and cellular endocrinology* **382**, 521–526, <https://doi.org/10.1016/j.mce.2013.10.008> (2014).
58. Mera, P. *et al.* Osteocalcin Signaling in Myofibers Is Necessary and Sufficient for Optimum Adaptation to Exercise. *Cell metabolism* **23**, 1078–1092, <https://doi.org/10.1016/j.cmet.2016.05.004> (2016).
59. Joiner, M., Le Toriell, E., Despouy, G. & Stern, M. H. The MTCP1 oncogene modifies T-cell homeostasis before leukemogenesis in transgenic mice. *Leukemia* **21**, 362–366, <https://doi.org/10.1038/sj.leu.2404476> (2007).
60. Gritti, C. *et al.* Transgenic mice for MTCP1 develop T-cell prolymphocytic leukemia. *Blood* **92**, 368–373 <http://www.bloodjournal.org/content/92/2/368.long?sso-checked=true> (1998).
61. Stern, M. H. *et al.* MTCP-1: a novel gene on the human chromosome Xq28 translocated to the T cell receptor alpha/delta locus in mature T cell proliferations. *Oncogene* **8**, 2475–2483 (1993).
62. Fisch, P., Forster, A., Sherrington, P. D., Dyer, M. J. & Rabbitts, T. H. The chromosomal translocation t(X;14)(q28;q11) in T-cell prolymphocytic leukaemia breaks within one gene and activates another. *Oncogene* **8**, 3271–3276 (1993).
63. Myllyharju, J. & Kivirikko, K. I. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends in genetics* **20**, 33–43, <https://doi.org/10.1016/j.tig.2003.11.004> (2004).
64. Canty, E. G. & Kadler, K. E. Procollagen trafficking, processing and fibrillogenesis. *Journal of cell science* **118**, 1341–1353, <https://doi.org/10.1242/jcs.01731> (2005).
65. Castillo-Briceño, P. *et al.* Collagen regulates the activation of professional phagocytes of the teleost fish gilthead seabream. *Molecular immunology* **46**, 1409–1415, <https://doi.org/10.1016/j.molimm.2008.12.005> (2009).
66. Castillo-Briceño, P., Arizcun-Arizcun, M., Meseguer, J., Mulero, V. & Garcia-Ayala, A. Correlated expression profile of extracellular matrix-related molecules during the inflammatory response of the teleost fish gilthead seabream. *Developmental and comparative immunology* **34**, 1051–1058, <https://doi.org/10.1016/j.dci.2010.05.007> (2010).
67. Castillo-Briceño, P. *et al.* A role for specific collagen motifs during wound healing and inflammatory response of fibroblasts in the teleost fish gilthead seabream. *Molecular immunology* **48**, 826–834, <https://doi.org/10.1016/j.molimm.2010.12.004> (2011).
68. Heino, J., Huhtala, M., Käpylä, J. & Johnson, M. S. Evolution of collagen-based adhesion systems. *The international journal of biochemistry & cell biology* **41**, 341–348, <https://doi.org/10.1016/j.biocel.2008.08.021> (2009).
69. Herr, A. B. & Farndale, R. W. Structural insights into the interactions between platelet receptors and fibrillar collagen. *The Journal of biological chemistry* **284**, 19781–19785, <https://doi.org/10.1074/jbc.R109.013219> (2009).
70. Schultz, G. S. & Wysocki, A. Interactions between extracellular matrix and growth factors in wound healing. *Wound repair and regeneration* **17**, 153–162, <https://doi.org/10.1111/j.1524-475x.2009.00466.x> (2009).
71. Fenton, R. A. & Knepper, M. A. Urea and renal function in the 21st century: insights from knockout mice. *Journal of the American Society of Nephrology* **18**, 679–688, <https://doi.org/10.1681/ASN.2006101108> (2007).
72. Fenton, R. A. Essential role of vasopressin-regulated urea transport processes in the mammalian kidney. *Pflügers Archiv* **458**, 169–177, <https://doi.org/10.1007/s00424-008-0612-4> (2009).
73. Ranade, K. *et al.* Genetic variation in the human urea transporter-2 is associated with variation in blood pressure. *Human molecular genetics* **10**, 2157–2164 (2001).
74. Tsai, H. J. *et al.* Genetic variants of human urea transporter-2 are associated with metabolic syndrome in Asian population. *Clinica chimica acta* **411**, 2009–2013, <https://doi.org/10.1016/j.cca.2010.08.025> (2010).
75. Ghosh, M., McAuliffe, B., Subramani, J., Basu, S. & Shapiro, L. H. CD13 regulates dendritic cell cross-presentation and T cell responses by inhibiting receptor-mediated antigen uptake. *The Journal of immunology* **188**, 5489–5499, <https://doi.org/10.4049/jimmunol.1103490> (2012).
76. Trapnell, C. *et al.* Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature protocols* **7**, 562–578, <https://doi.org/10.1038/nprot.2012.016> (2012).
77. Sun, J., Nishiyama, T., Shimizu, K. & Kadota, K. TCC: an R package for comparing tag count data with robust normalization strategies. *BMC Bioinformatics* **14**, 219, <https://doi.org/10.1186/1471-2105-14-219> (2013).

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Author Contributions

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Additional Information

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