

Circulating miRNAs involved in the immune response of the European seabass (*Dicentrarchus labrax*)

Elena Sarropoulou^{a,*}, Pantelis Katharios^a, Elisavet Kaitetzidou^a, Giuseppe Scapigliati^b, Andrea Miccoli^c

^a Institute of Marine Biology, Biotechnology, and Aquaculture, Hellenic Centre for Marine Research, Thalassocosmos, Heraklion, Crete, Greece

^b Department for Innovation in Biological, Agro-Food and Forest Systems, University of Tuscia, 01100, Viterbo, Italy

^c National Research Council, Institute for Marine Biological Resources and Biotechnology (IRBIM), 60125, Ancona, Italy

ARTICLE INFO

Keywords:

Circulating miRNA
Teleost
Immune system
Vaccination
Bacterial infection
European seabass

ABSTRACT

Understanding the immune response in fish through transcriptomic and microRNA (miRNA) profiling may unlock critical insights into disease resistance mechanisms. The objective of the present study was to examine the immune modulation of the European seabass (*Dicentrarchus labrax*) following bacterial infection and vaccination. Therefore, sequencing of circulating miRNA isolated from blood serum and 3'UTR transcriptome sequencing of head kidney was conducted. In the infected fish 19 miRNAs were found to be differentially expressed. This included two novel miRNAs exhibiting high levels in the infected fish. Regarding circulating miRNAs following vaccination, three specific miRNAs have been identified that demonstrated a substantial increase in expression. Two of them, miR-216b and miR-30a-5p, have been documented to possess the capacity to delay the progression of viral infections. 3'UTR sequencing analysis of the infected fish revealed no significant enrichment of down-regulated transcripts. However, there was a significant enrichment of up-regulated transcripts related to ribosome biogenesis and protein processing. In vaccinated fish up-regulated transcripts did not demonstrate substantial enrichment. Down-regulated genes on the other hand were involved in cytoskeleton organization and apoptosis, indicating that cellular disruption might be a potential hindrance to effective immunity. Overall, these results provide first insights into the progression and regulation of host immune responses to pathogen infection and vaccination. Moreover, the detection of in total 13 differential expressed circulating miRNAs, including regulators of critical innate immunity-related genes such as Toll-like receptor 18, suggests a potential for circulating miRNAs to play a significant role in the post-transcriptional control of fish immune defenses.

1. Introduction

Achieving efficient disease prevention is a major challenge in the life cycle of teleosts, particularly in the aquaculture sector [1]. A substantial body of research has been conducted on fish species of economic importance, such as the gilthead seabream (*Sparus aurata*) and the European seabass (*Dicentrarchus labrax*), which also contributed substantially to advancements in research at the molecular level (e.g., Refs. [2–4]). Extensive knowledge (e.g. Genome assembly, whole transcriptomes from lymphoid and non-lymphoid tissues, functional annotation databases) has been built up for both species over the last two decades, and particularly for the European seabass, the immune system has been extensively studied [2,5–8]. Indeed, pioneering works have

contributed to elucidating the teleost immune system. These works include, but are not limited to, transcriptome studies, reporting differentially expressed genes (e.g., Refs. [9–11]), as well as small non-coding RNA (snRNA) patterns for teleost immune response (e.g., Refs. [12–16]).

In the context of teleost immune response, mucosal surfaces constitute the primary line of defense against pathogens. These surfaces, which include skin, gills, gut, and olfactory organ are sites of significant molecular activity that plays a crucial role in innate immunity [17]. In the absence of bone marrow and lymph nodes, the main lymphoid tissues in fish are the thymus, the head kidney, the spleen, gill-associated lymphoid tissue (GIALT) or interbranchial lymphoid tissue (ILT) and the mucosa-associated lymphoid tissue [18]. Among these, the head kidney

This article is part of a special issue entitled: Med aqua animal health immune status published in Fish and Shellfish Immunology.

* Corresponding author.

E-mail address: sarris@hcmr.gr (E. Sarropoulou).

<https://doi.org/10.1016/j.fsi.2025.110232>

Received 7 November 2024; Received in revised form 31 January 2025; Accepted 23 February 2025

Available online 24 February 2025

1050-4648/© 2025 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

assumes hemopoietic functions and represents the primary immune organ where all leukocyte populations are found, and where antigen processing and formation of IgM and immune memory through melanomacrophage centers occur. The blood on the other hand contains the majority of the leukocyte populations that are recognized in vertebrates, as well as soluble IgM, which constitutes the predominant systemic antibody class in fish.

Along with the high diversity of teleosts, also a broad range of pathogens exists and major losses have been documented in several farmed finfishes. Although to present days extensive research has been conducted to investigate fish immune responses from both physiological and molecular perspectives, reliable non-lethal early detection methods for efficient diagnosis and prognosis in fish are still lacking. The evaluation of expression patterns of marker transcripts, whole transcriptome, or enzymatic activities, as informative as they might be, require fish specimens to be sacrificed. The ultimate challenge in the field appears to be the development of non-lethal and broadly applicable methodologies to investigate potential transcriptomic responses, especially for fish species of commercial interest. The study of circulating miRNAs in teleost fish as potential non-lethal biomarkers is a rapidly emerging field of research. One of the earliest studies examined the association between circulating miRNAs and vitamin K levels, with a focus on their role in reproductive function in the Senegalese sole (*Solea senegalensis*) [19]. Subsequent research investigated the relationship between circulating miRNAs and nutritional and environmental stressors in the rainbow trout (*Oncorhynchus mykiss*), with a view to identifying biomarkers of stress and sex [20,21]. With regard to the European seabass, to the best of our knowledge, only one study has been published that deals with circulating miRNAs. This study reports the association with gender and stress response. The authors identified five potential miRNAs related to the stress response and three related to gender [22]. This finding indicates that only a limited number of circulating miRNAs may function as potential biomarkers. Nonetheless, investigations are still in their nascent stages and have not yet been implemented. In medical research, cell-free-circulating miRNAs were already recognized as important emerging biomarkers in body fluids for various complex human diseases and it has now been established that non-coding RNA molecules exert post-transcriptional control over stability and translation of vast networks of protein-coding genes, during both early embryonic development and in adult cell types [23]. Although miRNA regulation of immune system genes has been extensively studied in higher vertebrates, particularly in humans [24], there is much less information on how miRNAs regulate the immune response in teleost fish. Some studies have identified specific miRNAs with distinct expression patterns following immune challenges i.e. [15,25,26], however, data on miRNA regulation in teleosts, especially in the European seabass, remains limited, particularly regarding the role of circulating miRNAs in immune response regulation. Consequently, exploring teleost circulating miRNAs and the corresponding immune response outcomes holds great potential for providing not only a better understanding of disease pathology but also a foundation for developing non-lethal methods for early disease detection. In the present study, we have examined circulating miRNA in the blood serum of the European seabass along with gene expression patterns in the head kidney after vaccination as well as after infection with *Aeromonas veronii* bv. *Sobria*, an emerging pathogen of the European seabass cultured in the Mediterranean Sea associated with severe losses [27,28]. Identified miRNAs represent the first candidates in European seabass to serve as putative non-lethal biomarkers for disease detection, as early diagnosis of disease provides the opportunity to take immediate action and to prevent the disease from spreading and infecting other organisms.

2. Material and methods

All procedures involving fish handling were conducted following the guidelines provided by authorized personnel (FELASA-accredited

certification) as well as the “Guidelines for the treatment of animals in behavioral research and teaching” [29], the Ethical Justification for the use and treatment of fishes in research: An update, and the “Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 on the protection of animals used for scientific purposes” [30].

2.1. Fish and experimental conditions

Experiment 1: Healthy European seabass, weighing approximately 250 g were experimentally challenged with *Aeromonas veronii* bv. *sobria*, strain PDB, as previously described [31]. Briefly, the fish were immersed in a bacterial solution derived from an overnight culture, at a final concentration of 10^5 CFU/mL, for 2.5 h. After exposure, the fish were transferred to 250 L tanks equipped with autonomous filtration systems and monitored for 10 days. In total 10 fish were challenged with the pathogen. Sampling was done at day 3 post challenge when most fish displayed typical clinical signs of the disease including anorexia, jaundice, and hemorrhaging. The fish were anesthetized in MS222 and following blood sampling ($n = 4$) they were sacrificed in an overdose of the anesthetic. Head kidney of the same individual as of the blood sampling was removed aseptically and placed immediately in RNAlater (Qiagen, Duerren, Germany). Fish were not fed during the challenge period, and anesthesia was achieved using tricaine methanesulfonate (MS222, Sigma-Aldrich Inc., St Louis, MO, USA, #E10521). An equal number of healthy European seabass (*Dicentrarchus labrax*) with comparable weight and not challenged with the pathogen were sampled as control fish from HCMR facilities. The experimental protocol was approved by the competent authority (protocol number 147115, July 17, 2017).

Experiment 2: Fish that were not subjected to any vaccination and/or viral infection and which resulted nodavirus-negative to a PCR screen served as the control experimental group ($n = 4$, length 24.1 ± 0.68 cm, gutted weight 133 ± 5.08 g). Nodavirus-vaccinated fish received the Pharmaq ALPHA JECT micro® 1Noda vaccine (inactivated Red-spotted Grouper Nervous Necrosis Virus RGNNV ≥ 0.07 antigenicity units per dose in emulsion for injection, adjuvated with liquid paraffin) by intraperitoneal injection (IP) on December 23rd 2019 and were sampled on February 4th 2020 ($n = 4$, length 25.2 ± 1.30 cm, gutted weight 159.65 ± 10.85 g). Onset of immunity is reported after 466-degree days with the mean water rearing temperature at 19.4°C . Samples were taken from non-vaccinated and vaccinated individuals.

2.2. Total RNA extraction from fish serum and head kidney

European seabass blood was collected via caudal venipuncture into 2 mL tubes, using 25G needles paying particular attention to avoiding hemolysis. Blood was left undisturbed at room temperature for 4 h and clots were removed by centrifugation at $2000 \times g$ for 10 min, the supernatant was removed and shock-frozen in liquid nitrogen. Total RNA was extracted from the serum by applying the QIAzol (Qiagen, Germany) extraction protocol. In brief, 5 vol of QIAzol were added to one volume of fish serum and vortexed. After adding an equal volume of chloroform, the upper aqueous phase was collected and 1.5 vol of 100 % EtOH were added and mixed thoroughly by pipetting. Samples, including any precipitate, were transferred into a RNeasy Mini column (miRNeasy, Qiagen, Duerren, Germany) followed by washing and precipitation steps as recommended by the manufacturer. The final elution volume was set to 30 μL of RNase-free water and the total RNA was evaluated by RNA picochip (DNAnalyzer, Agilent).

Total RNA was extracted from the head kidneys using the Nucleospin miRNA kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. In brief, the head kidney was disrupted with a mortar and pestle in liquid nitrogen and homogenized in lysis buffer by passing the lysate through a 23-gauge (0.64 mm) needle five times. The quantity of extracted RNA was estimated utilizing a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The

quality of the RNA was additionally assessed through agarose (1 %) gel electrophoresis and capillary electrophoresis using the RNA Pico Bioanalysis chip (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA 95051, USA). Samples with an RNA integrity number value between 8.9 and 9.9 were deemed suitable for library construction.

2.3. sncRNA library construction and sequencing

sncRNA libraries were prepared using the NEBnext multiplex Small RNA Library Preparation kit for Illumina sequencing (New England Biolabs, Ipswich, MA, USA) with 18 ng of isolated serum RNA as starting material. According to the manufacturer's recommendation, size fractionation was carried out by electrophoresis of a polyacrylamide gel (6 % TBE gel, Lonza, Basel, Switzerland) at 4 °C for 1 h. Different multiplex identifier tags provided by NEB were added to each sample. sncRNA libraries were evaluated by DNA high-sensitivity chips (Bioanalyzer, Agilent) and quantified by Qubit (Life Technologies, Carlsbad, CA, USA) measurements. Differentially indexed libraries were pooled at a concentration of 4 nM and single-strand sequenced on an Illumina NextSeq500 sequencing platform at the Genomics Facility of the Institute of Molecular Biology & Biotechnology, Forth, Crete, Greece.

2.4. 3'UTR library construction and sequencing

3'UTR libraries were prepared from 500 ng of head kidney total RNA using Lexogen's QuantSeq 3' mRNA-Seq Library Prep Kit REV for Illumina, according to the manufacturer's instructions. Sequencing (1 × 75 single-end strategy) was conducted on an Illumina NextSeq500 platform at the Genomics Facility of the Institute of Molecular Biology & Biotechnology, Forth, Crete, Greece.

2.5. Sequencing data processing

Quality control of all reads (mRNA and sncRNA) was achieved using Fastqc v0.10.0 software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) prior to and following trimming using Trimmomatic v0.3 [32] to remove residual adapters, low-quality nucleotides (nt). Trimmed reads were imported into CLC Genomics Workbench (v10.1). Putative sncRNAs including all types of sncRNA were extracted, reads were counted accordingly, and the minimum sampling count was set to 10. Putative miRNAs were annotated by mapping sequencing reads against the miRbase (release 21.1) available teleost miRNAs in the following order, *Astatotilapia burtoni*, *Oryzias latipes*, *Tetraodon nigroviridis*, *Fugu rubripes*, *Danio rerio*, *Cyprinus carpio*, *Gadus morhua*, *Hippoglossus hippoglossus*, *Paralichthys olivaceus*, *Ictalurus punctatus*, *Salmo salar*, *Petromyzon marinus*, as well as miRNAs from *Gorilla*, *Homo sapiens*, and *Mus musculus* [33], and against the three-spined stickleback (*Gasterosteus aculeatus*, non-coding RNA Ensembl resource *Gasterosteus aculeatus*.BROADS1.ncrna with the default parameters of CLC workbench. Already a decade ago, strong evolutionary relationships have been established between the European seabass and the three-spined stickleback by synteny analysis and collinearity [22,34,35]. Subsequently, variants of the same miRNAs were merged, resulting in a list of "sampled grouped" transcripts with the corresponding read counts. Putative novel miRNAs were annotated by running the mir-Deep2 software (mirdeep2-0.1.3) on previously unannotated transcripts with total read counts higher than 500.

3'UTR reads were imported to CLC workbench and mapped onto the European seabass genome v1.0 (GCA_000689215.1). Read counts were obtained and employed for differential gene expression analysis employing a negative binomial generalized linear model using the R DESeq2 package.

2.6. Differential expression analysis

Differential expression (DE) analysis for both miRNA and mRNA was

assessed by DESeq2 implemented in SarTools vs 1.2.0 [36] with default parameters. The control group from each experiment was set as reference level respectively. Transcripts with $p\text{-adj.} < 0.05$ or $p\text{-value} < 0.005$, and fold change (FC) $> |1|$ were considered as differentially expressed. Heatmaps, volcano plots, as well as PCA analysis, were carried out in R [R Core Team 2017]. Enrichment analysis were carried out applying the gProfiler g:GOST algorithm and visualization tool.

2.7. Identification of mRNAs targeted by the most abundant, and differentially expressed miRNAs

Putative mRNA targets of the most abundant, differentially expressed, and characterized miRNAs were identified using RNAhybrid, v.2.12 [37], with the energy threshold of the hybridization dynamics set to minimum free energy (mfe) ≤ -20 as well as TargetScan Perl script [38] with customized European seabass 3'UTR. Subsequently, only 3'UTRs with perfect complementary base-pairing to the miRNA seed region (base pairs 2–7) were taken into consideration. The complete 3'UTRs of the European seabass were retrieved from the Ensembl database.

3. Results

3.1. sncRNA and mRNA data generation and evaluation

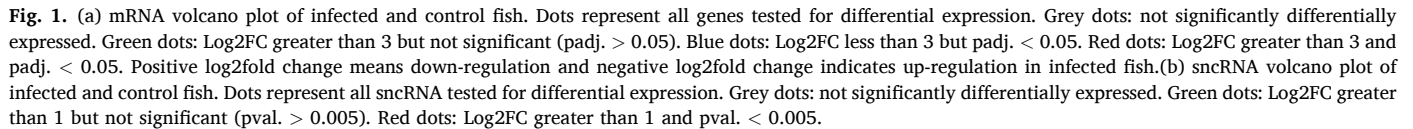
Sequencing of all sncRNA libraries resulted in more than 320 million reads with an average of about 20 million trimmed reads per sample. Plotting the obtained sncRNA read lengths displayed the typical peak at 19–24 nt, which corresponds to the read length of miRNAs.

3'UTR libraries generated about 10 million reads, with an average successful mapping rate onto the European seabass genome of ~75 % (Suppl. Table 1). All sequencing data have been submitted to NCBI under the BioProject accession number PRJNA1174768.

3.2. Gene and miRNA expression analysis in infected fish (experiment 1)

A total of 343 genes were identified as differentially expressed in infected compared to control fish, with 161 being up-regulated and 187 being down-regulated in infected fish (Fig. 1a, Suppl. Table 2). Table 1 presents the ten most significant genes being either up or down-regulated. Among the significantly differentially expressed genes, ENS-DLAG00005003529 exhibited the highest read abundance and was found to be down-regulated in the head kidney of the infected fish. This is a protein-coding gene encoding for hemoglobin, beta adult 1 (*hba1*) (NCBI accession number XM051389353.1), as per the best NCBI blastn match (e-value = 0.0). The gene with the highest read count being up-regulated in infected fish was found to be the heat shock protein family member 5 (*hspa5*). The transcripts being up- and down-regulated the most in terms of fold change in the infected fish were ENS-DLAG00005002533 (uncharacterized protein as per NCBI blastn match, with e-value = 0.0 and accession number XM_051399412.1) and troponin T type 3a (*tmt3a*) respectively. Fig. 1a illustrates mRNA transcripts, being in the infected fish either up- (negative log2fold change) or down-regulated (positive log2fold change). Enrichment analysis revealed four Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, eight transcription factors and 12 Biological Process (BP) GO terms to be significantly enriched for the genes being down-regulated in infected fish (Fig. 2).

Regarding microRNA expression 19 miRNAs with $p\text{-value} < 0.005$ were found and 11 with $p\text{-adj} < 0.05$ (Fig. 1b–Suppl. Table 3). Among the differentially expressed miRNA three miRNAs, i.e. CAJNNU010000002.1, ENSGACT00000029667.1, CAJNNU010000023.1 were found to be novel, not yet described. The latter two are higher expressed in infected fish while CAJNNU010000002.1 has a higher read count abundance in the control fish. It is noteworthy that one of the novel miRNAs (CAJNNU010000023.1, Fig. 3a), exhibit a



List of 10 most up or down-regulated genes of experiment 1 as per statistical support (padj). The log₂ fold changes of the up regulated genes in the infected fish are in bold.

The role of circulating miRNAs in teleost fish is of great importance for a highly coordinated and efficient immune response [19], as they serve as pivotal regulators, modulating a plethora of immune system functions and signaling pathways in response to pathogenic infections [39]. Recent studies have highlighted the involvement of miRNAs in critical processes such as cytokine signaling, antigen presentation, and

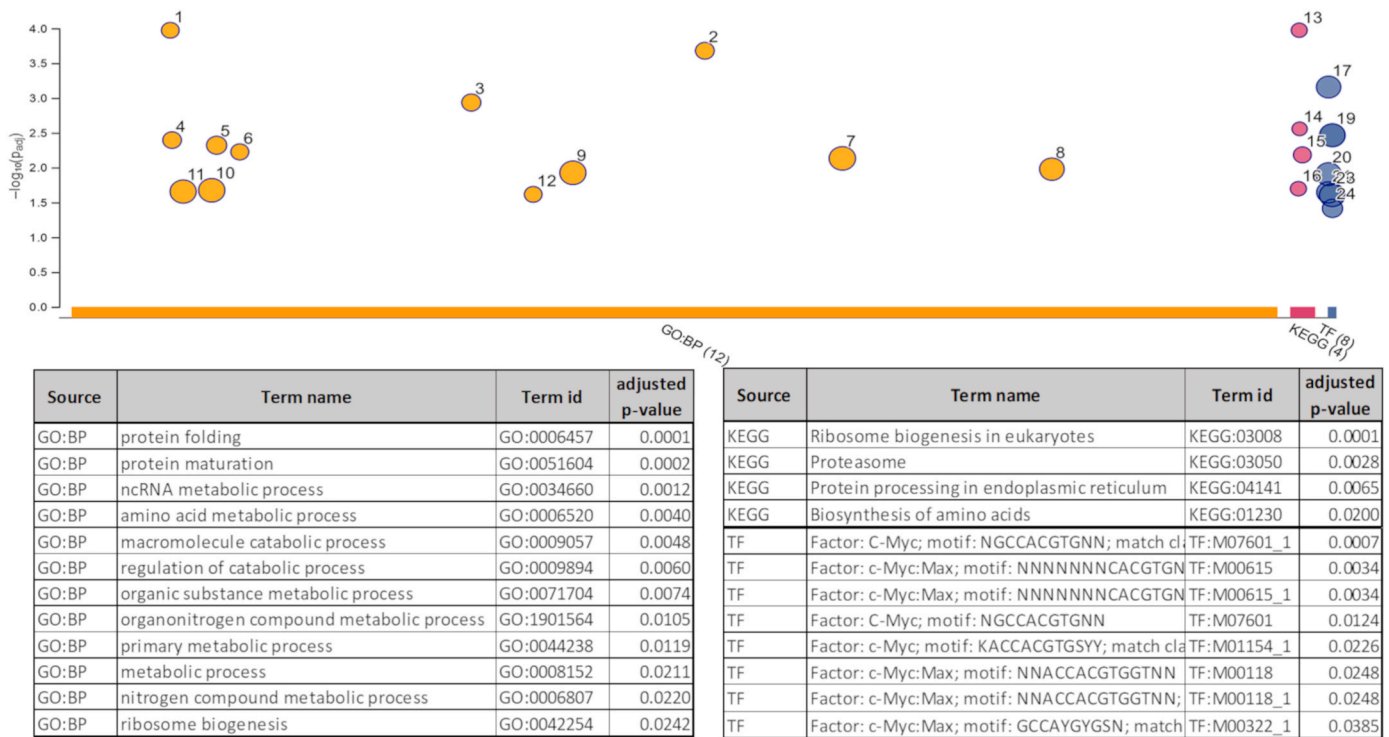


Fig. 2. Enrichment analysis conducted on significantly up-regulated genes ($\log_2FC > 1$, $p_{adj} < 0.05$) in infected fish. Graphs were obtained applying the gProfiler g:GOST algorithm and visualization. The dots indicate the Gene Ontologies categories as well as KEGG terms that passed the p-adjustment threshold value and therefore appeared to be significantly enriched within the set of differentially expressed genes.

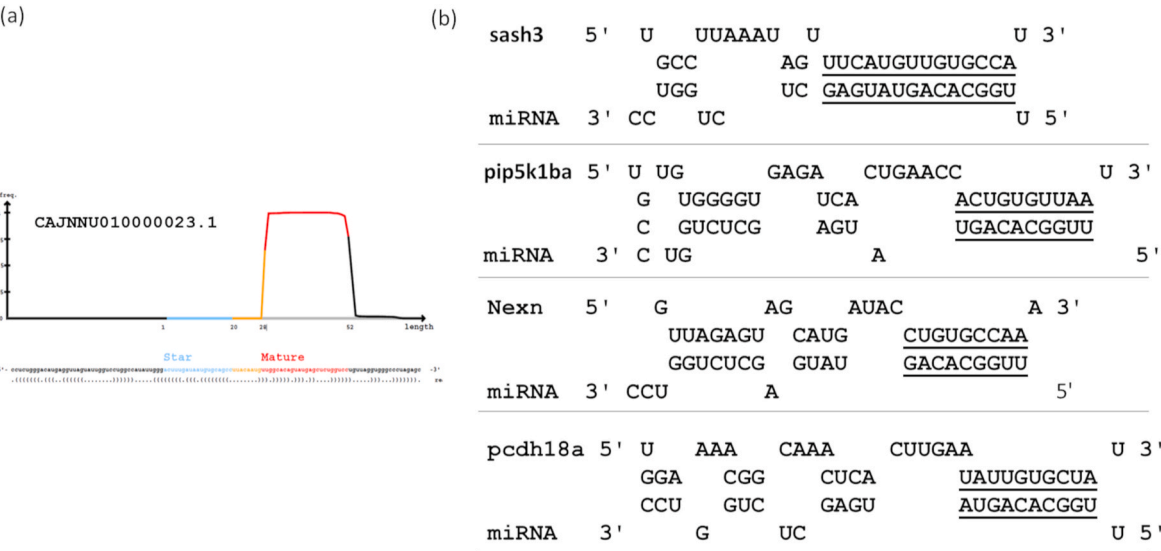


Fig. 3. (a) Structure of the putative novel miRNA CAJNNU010000023.1 obtained by running the mirDeep2 software (mirdeep2-0.1.3). The seed region is underlined, the Mature sequence is highlighted in red and the star is in blue. (b) Hybridization of CAJNNU010000023.1 with 8mer alignment of the seed region (by RNAhybrid-2.1.2) to four targets found to be down-regulated in infected fish.

the regulation of inflammatory responses, underscoring their significance in both innate and adaptive immunity within these aquatic species [15,40]. The present study aimed to detect circulating miRNAs that may play a role in immune response. Additionally, it examined the gene expression patterns in the head kidney of the European seabass, an aquaculture-relevant species that has gradually but consistently been established since 1994 as a marine model species in immunology [7]. Therefore, circulating blood miRNAs were identified, along with comparisons of the integrated transcriptome response in the head kidney.

The 3'UTR RNA-Seq method was selected, as this method has been shown to be more accurate and at a lower cost. Particularly, at lower read depths, a focus on the 3' end results in greater stability of differential gene expression assessments [41], and since the entire European seabass genome is available, it was possible to proceed with 3' UTR sequencing. On average, more than 10 million reads were obtained for each biological condition. The latter ensures an appropriate representation of genes containing putative isoforms. In terms of miRNA sequencing, an average of 20 million reads were obtained for each

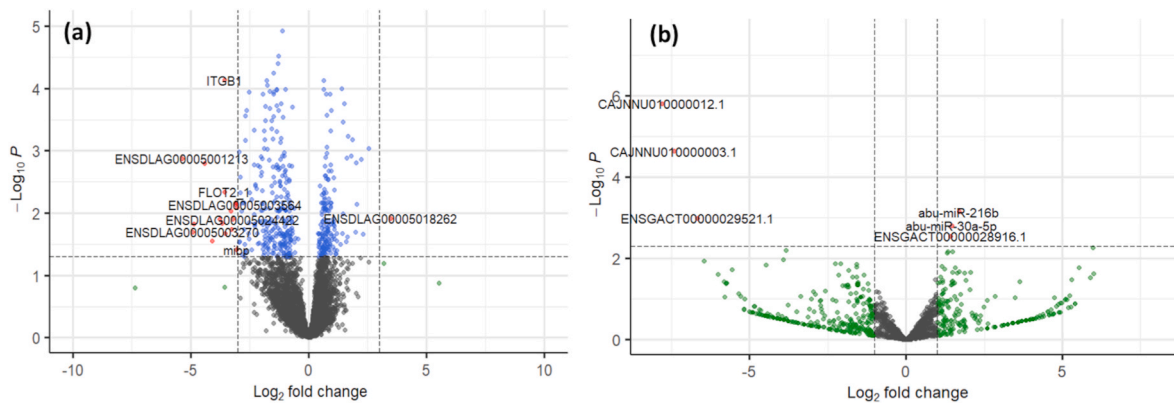


Fig. 4. (a) mRNA volcano plot of vaccinated fish. Dots represent all genes tested for differential expression. Grey dots: not significantly differentially expressed. Green dots: Log2FC greater than 3 but not significant (padj. > 0.05). Blue dots: Log2FC less than 3 but padj. < 0.05. Red dots: Log2FC greater than 3 and padj. < 0.05. (b) sncRNA volcano plot of vaccinated fish. Dots represent all sncRNA tested for differential expression. Grey dots: not significantly differentially expressed. Green dots: Log2FC greater than 1 but not significant (pval. > 0.005). Red dots: Log2FC greater than 1 and pval. < 0.005.

sample. In order to achieve differential expression (with high fold changes), a typical recommendation is to have between 1 and 5 million mapped reads per sample [42]. Conversely, for the discovery of novel miRNAs, more extensive sequencing is necessary, with 5–10 million reads per sample recommended [43]. Accordingly, the present study addresses the aforementioned requirements for novel miRNA discovery as well as for differential expression assessments.

No significant gene set enrichment resulted from 3'UTR analysis of down-regulated transcripts in infected fish and up-regulated transcripts in vaccinated fish had been detected. Instead, up-regulated transcripts in infected fish revealed an enrichment of 12 GO Biological process terms with the majority related to protein processing and ribosome biogenesis, along with enriched motives of transcription factor c-Myc and four KEGG pathways being again involved in the ribosome biogenesis and diverse protein processes. Similarly, in the East Asian fourfinger threadfin fish (*Eleutheronema tetradactylum*) head-kidney-specific differentially expressed genes (DEGs) were also enriched in the KEGG pathway "Ribosome biogenesis in eukaryotes" [44]. In that context, *Streptococcus iniae*, a pathogen frequently isolated from the brains and head kidneys of infected fish, presented a relevant case; the authors hypothesized that the up-regulation of genes related to ribosome biogenesis in the head kidney following infection may suppress immune defense mechanisms, potentially facilitating a higher bacterial load of *S. iniae* in the head kidney. This could suggest a potential trade-off between ribosome production and immune system efficiency, where increased ribosome biogenesis might compromise the fish's ability to mount an effective immune response. It is noteworthy that the relationship between ribosome biogenesis and innate immunity has also been the subject of investigation in several other studies (e.g. Refs. [45, 46]). Ribosomes are paramount to a multitude of processes ensuring homeostasis in organisms [47]. While ribosomal biogenesis is effectively essential for protein production, and therefore cell growth, proliferation, and animal development, increasing evidence is accumulating as to the consequences of both deregulated over-production and haploinsufficiency of ribosomal biogenesis to tumorigenesis [48]. The growth-promoting factor C-Myc is one of the oncoproteins displaying regulatory roles in ribosomal biogenesis [49] and induces ribosome biogenesis by stimulating PolI, Pol II, and Pol III transcription [50]. Studies have further revealed that rRNA accumulation and/or nucleolar activity regulate double-stranded (ds) DNA-sensing, which in turn restricts virus reproduction and regulates inflammation [45]. Given the considerable energy expenditure associated with ribosome formation, it is maybe unsurprising that this process is integrated into vital cellular responses that determine survival, including proliferation, lifespan, and cellular immunity.

In vaccinated fish, 18 down-regulated genes were significantly

enriched in the Biological Process GO terms mainly related to cytoskeleton biogenesis, organization, and polymerization, as well as two KEGG pathways, namely "Regulation of actin cytoskeleton" and "Apoptosis". Similarly, in turbot (*Scophthalmus maximus*), genes associated with cell adhesion and the cytoskeleton, as well as apoptosis, were observed to exhibit differential expression following the IP administration of vaccines containing *Philasterides dicentrarchi* antigen differing in terms of adjuvants [51]. In that case, intense cell traffic to and from the peritoneal cavity, including free cells, cells attached to the mesothelium, and others migrating to lymphoid organs was observed, but, with regards to transcriptional patterns, the authors reported a generally strong up-regulation of genes underlying the above processes and pathways in vaccinated fish. Additionally, while both, the "Regulation of actin cytoskeleton" and "Apoptosis" KEGG pathways were identified as significantly enriched in the aforementioned and in the present study, the mapping genes differed. The disparate transcriptomic findings may be attributed to the distinct adjuvants employed. In turbot chitosan-PVMA microspheres, Freund's complete adjuvant, aluminum hydroxide gel or Matrix-Q (Isconova, Sweden) were used as adjuvants, while in the present study the vaccine's adjuvant was liquid paraffin (mineral oil). The lesions in the peritoneal cavity, including granulomas and adhesions between organs or the organs and the peritoneal wall, possibly caused by vaccines [52], may vary depending on the adjuvant. Moreover, a significant proportion of the free cells present within the peritoneal cavity demonstrate an affinity for the vaccine, leading to the formation of masses that become attached to the peritoneal wall. Consequently, the generation of cell-vaccine masses necessitates the robust expression of genes associated with cell-cell or cell-matrix adhesion and cell-cell junctions, as well as those involved in the actin cytoskeleton. The cytoskeleton plays a pivotal role in regulating differentiation, polarization, migration, and adhesion processes, which are essential for innate immunity and cellular self-defense [53]. Actin dynamics are essential for the activation of immune cells, particularly T cells and macrophages. Hence, the down-regulation of pathways involved in actin regulation may impair these cells' ability to form immunological synapses and properly engage with antigens. This could result in a weaker activation signal, potentially leading to a reduction in the efficacy of the vaccine. The down-regulation of the "Regulation of Actin Cytoskeleton" pathway after vaccination therefore suggests a potential compromise in the teleost immune response, affecting essential processes such as cell migration, activation, cytokine secretion, and intercellular interactions. An understanding of these changes can facilitate the optimization of vaccination strategies for enhanced immune outcomes in teleosts.

With regards to the identified circulating microRNAs undergoing regulation in infected fish, a total of 19 microRNAs exhibited differential

Table 2

List of 10 most up or down-regulated genes of experiment 2 as per statistical support (padj). The log₂ fold changes of the up regulated genes in the vaccinated fish are in bold.

Id	CPBL Mean	VPBL Mean	log ₂ fold change	padj	Encoded protein annotation
btg1	414	94	−2.1	3.52E-11	BTG Anti-Proliferation Factor 1, interacts with several nuclear receptors, and functions as a coactivator of cell differentiation
HSPA8_2	331	86	−1.941	8.224E-11	Heat Shock Protein Family A (Hsp70) Member 8, functions as a chaperone and binds to nascent polypeptides to facilitate correct folding
ENSDLAG00005019812	127	15	−3.071	2.02E-10	Ubiquitin A-52 residue ribosomal protein fusion product 1, involved in protein catabolic processes
fth1a	30284	3013	−3.271	2.50E-08	Ferritin Heavy Chain 1, contributes to storage of iron in a soluble and nontoxic state
atg3	107	37	−1.54	3.44E-08	Autophagy-Related Protein 3, ubiquitin-like-conjugating enzyme, a component of ubiquitination-like systems involved in autophagy
atp5mc1	62	165	1.431	3.44E-08	ATP Synthase Membrane Subunit C Locus 1, a subunit of mitochondrial ATP synthase that catalyzes ATP synthesis
rps24	652	218	−1.579	5.97E-08	Ribosomal Protein S24, a component of the 40S subunit
kdm5ba	122	46	−1.392	5.97E-08	Lysine Demethylase 5B, demethylates lysine 4 of histone H3 for transcriptional repression, and may also play a role in DNA repair
si:dkey-56i24.1	618	1257	1.066	1.02E-07	Glycosyltransferase family 92, transfers glycosyl groups
spns3	372	705	0.912	3.11E-07	SPNS lysolipid transporter 3, sphingosine-1-phosphate, predicted to be involved in sphingolipid transport and transmembrane transport

expression, comprising two novel microRNAs that have not yet been characterized. The miRNA differential expression in vaccinated fish amounted to a total of five miRNAs, two of which were novel. While innate immune pathways triggered by viral or bacterial infections viruses and extracellular bacteria are generally considered to be distinct, some mechanisms were demonstrated to be triggered by both pathogen types [54] our data did not highlight this, as there were no common differentially expressed miRNAs between infected and vaccinated fish. It is reasonable to expect that the anticipated number of differentially expressed circulating microRNAs may remain low when comparing the two conditions. A study of rainbow trout, for example, investigated the circulating miRNAs between dissolved oxygen (DO) levels (normoxia and hypoxia) and diet composition (low-carbohydrate (LC) or high-carbohydrate (HC)). In total, 252 circulating miRNAs were identified, of which only four were differentially expressed (with threshold criteria p-value <0.05 and log₂ fold change >0.58) between the control fish and the treated ones [20]. It can thus be concluded that the current findings have significant scientific value. A further focus of the present study has been the microRNAs with higher expression levels, as well as those with no or low expression levels in the control or treated fish, respectively. Markedly one of the novel miRNAs (CAJNNU01000023.1, Fig. 3a) has been found to be a putative regulator of 38 genes belonging to immune-related categories. Among them *toll like receptor 18* (*tlr18*), an extensively expressed fish-specific TLR belonging to the TLR1 family (Suppl. Table 5). TLRs are essential pattern recognition receptors (PPRs) that can identify conserved pathogen-associated molecular patterns (PAMPs). Moreover, it has also been reported that TLR18 is up-regulated in isolated peripheral blood lymphocytes of yellow catfish after stimulation with lipopolysaccharide (LPS), peptidoglycan (PGN), and polyinosinic:polycytidylic acid (poly (I:C)) [55].

CAJNNU01000023.1 has further been detected to hybridize (seed region, 8mer) to four genes significantly down-regulated in infected fish (Fig. 3b). One of the four genes, *sash3* has been recently described in humans to play a significant role in T cell proliferation, activation and survival. The loss of function in human has been associated with immunodeficiency and may negatively affect T-cell function [56]. The available evidence on the role of *sash3* in teleosts is currently limited. However, its evolutionary conservation suggests that it may also be involved in analogous immune processes in fish. The highest expressed gene was ENSDLA000000329, annotated via NCBI as hemoglobin, beta adult 1 (*hbba1*) and down-regulated in the infected fish revealed 8mer hybridization with miR-30e-3p which was found to be up-regulated after infection (p-value <0.005). In *Megalobrama amblycephala*, studies have demonstrated a reduction in the expression levels of Hbβ (as well as Hbα) following infection. This could suggest a potential correlation between haemoglobin function and the physiological responses to pathogens. The observed down-regulation may also be associated with damage to red blood cells or haematopoietic organs during infections [57].

Concerning miRNAs being up-regulated in vaccinated fish of particular interest are the miR-216b and miR-30a-5p. The former was reported to have the potential to exert an inhibitory effect on the expression of viral genes. It is also possible that this miRNA may target host genes that are involved in antiviral signalling pathways [15]. As for the latter miRNA, miR-30a-5p much less information is available. Yet in the miiuy croaker (*Miichthys miiuy*) miR-30a-5p was down-regulated after poly (I:C) stimulation [58], even though, its mature sequence matches that of miR-30e-5p, which has been documented to be up-regulated in mammals in response to viral infections [59].

Overall, these findings, obtained for one of the most relevant marine aquaculture species in Europe that is becoming a marine model species in the field of comparative immunobiology, contribute to a deeper understanding of the molecular basis of immune responses in teleosts. A

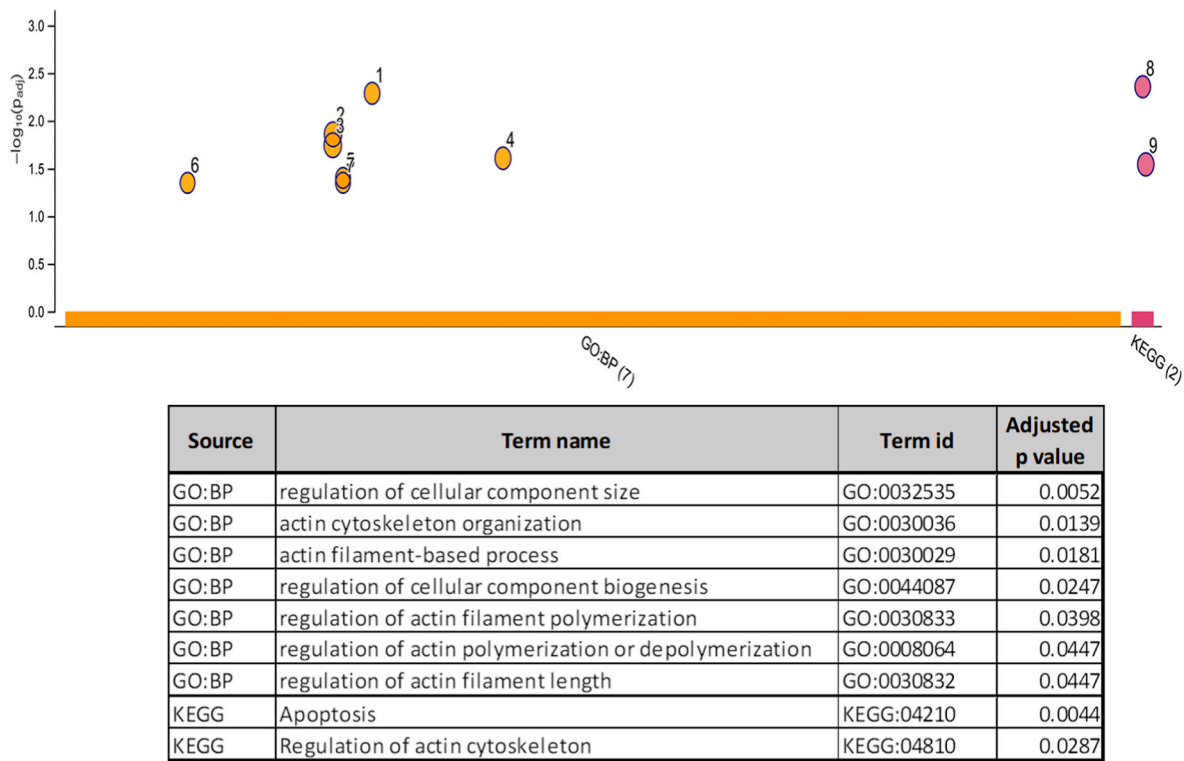


Fig. 5. Enrichment analysis of genes being significantly down-regulated (log2fold >1, padj <0.05) in vaccinated fish. Enrichment analysis were carried out applying the gProfiler g:GOST algorithm and visualization tool. The dots indicate the Gene Ontologies categories as well as KEGG terms that passed the p-adjustment threshold value and therefore appeared to be significantly enriched within the set of differentially expressed genes.

more thorough understanding of host immune regulation against bacterial infection or vaccination against NNV was achieved by elucidating the signatures and possible interactions between transcriptional and post-transcriptional regulatory mechanisms, paving the way to the development of more effective vaccination strategies aimed at improving disease resistance in aquaculture. Future efforts may be devoted to elucidating the affected pathways and, most importantly, their implications for fish health management.

Acknowledgments

The authors would like to extend their gratitude to the Genomics Facility of the Institute of Molecular Biology & Biotechnology, Forth, Crete, for their invaluable assistance. Furthermore, the authors express their gratitude to the IMBBC IT team and the computational resources, which were provided by IMBBC (Institute of Marine Biology, Biotechnology, and Aquaculture of the HCMR (Hellenic Centre for Marine Research)), for conducting this research. The establishment of the IMBBC HPC was made possible by financial support from the MARBI-GEN (EU Regpot) project, the LifeWatch Greece RI, and the CMBR (Centre for the Study and Sustainable Exploitation of Marine Biological Resources) RI. The authors would also like to thank Francesco Pascoli and Anna Toffan of Istituto Zooprofilattico Sperimentale delle Venezie for their support in experimental design.

This research was funded by the internal program SUPPGEN of IMBBC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2025.110232>.

Data availability

Data will be made available on request.

References

[1] C. Uribe, H. Folch, R. Enriquez, G. Moran, Innate and adaptive immunity in teleost fish: a review, *Vet. Med.* 56 (2011) 486–503, <https://doi.org/10.17221/3294-VETMED>.

[2] E. Sarropoulou, P. Sepulcre, L. Poisa-Beiro, V. Mulero, J. Meseguer, A. Figueras, B. Novoa, V. Terzoglou, R. Reinhardt, A. Magoulas, G. Kotoulas, Profiling of infection specific mRNA transcripts of the European seabass *Dicentrarchus labrax*, *BMC Genom.* (2009), <https://doi.org/10.1186/1471-2164-10-157>.

[3] E. Sarropoulou, J. Galindo-Villegas, A. García-Alcázar, P. Kasapidis, V. Mulero, Characterization of European sea bass transcripts by RNA SEQ after oral vaccine against *V. anguillarum*, *Mar. Biotechnol.* 14 (2012) 634–642, <https://doi.org/10.1007/s10126-012-9466-z>.

[4] E. Kaitetzidou, J. Xiang, E. Antonopoulou, C.S. Tsigenopoulos, E. Sarropoulou, Dynamics of gene expression patterns during early development of the European seabass (*Dicentrarchus labrax*), *Physiol. Genom.* 47 (2015) 158–169, <https://doi.org/10.1152/physiolgenomics.00001.2015>.

[5] G. Scapigliati, N. Romano, F. Buonocore, S. Picchietti, M.R. Baldassini, D. Prugnoli, A. Galice, S. Meloni, C.J. Secombes, M. Mazzini, L. Abelli, The immune system of sea bass, *Dicentrarchus labrax*, reared in aquaculture, *Dev. Comp. Immunol.* (2002), [https://doi.org/10.1016/S0145-305X\(01\)00057-X](https://doi.org/10.1016/S0145-305X(01)00057-X).

[6] S. Picchietti, A. Miccoli, A.M. Fausto, Gut immunity in European sea bass (*Dicentrarchus labrax*): a review, *Fish Shellfish Immunol.* 108 (2021) 94–108, <https://doi.org/10.1016/j.fsi.2020.12.001>.

[7] A. Miccoli, F. Buonocore, S. Picchietti, G. Scapigliati, The sea bass *Dicentrarchus labrax* as a marine model species in immunology: insights from basic and applied research, *Aquac. Fisherman (Syd.)* 9 (2024) 136–143, <https://doi.org/10.1016/j.aaf.2021.09.003>.

[8] A. Miccoli, V. Pianese, C. Bidoli, A.M. Fausto, G. Scapigliati, S. Picchietti, Transcriptome profiling of microdissected cortex and medulla unravels functional regionalization in the European sea bass (*Dicentrarchus labrax*) thymus, *Fish Shellfish Immunol.* 145 (2024) 109319, <https://doi.org/10.1016/j.fsi.2023.109319>.

[9] E. Chaves-pozo, S. Liarte, L. Fern, E. Abell, Pattern of expression of immune-relevant genes in the gonad of a teleost, the gilthead seabream, *Sparus aurata* L.) 45 (2008) 2998–3011, <https://doi.org/10.1016/j.molimm.2008.01.018>.

[10] K. Mitter, G. Kotoulas, A. Magoulas, V. Mulero, M.P. Sepulcre, A. Figueras, B. Novoa, E. Sarropoulou, Evaluation of candidate reference genes for QPCR during

- ontogenesis and of immune-relevant tissues of European seabass (*Dicentrarchus labrax*), Comp. Biochem. Physiol. B-Biochemistry Mol. Biol. (2009), <https://doi.org/10.1016/j.cbpb.2009.04.009>.
- [11] E. Sarropoulou, J. Galindo-Villegas, A. García-Alcázar, P. Kasapidis, V. Mulero, Characterization of European sea bass transcripts by RNA SEQ after oral vaccine against *V. anguillarum*, Mar. Biotechnol. (2012), <https://doi.org/10.1007/s10126-012-9466-z>.
 - [12] A. Ordas, Z. Kanwal, V. Lindenberg, J. Rougeot, M. Mink, H.P. Spaink, A.H. Meijer, MicroRNA-146 function in the innate immune transcriptome response of zebrafish embryos to *Salmonella typhimurium* infection, BMC Genom. 14 (2013) 1, <https://doi.org/10.1186/1471-2164-14-696>.
 - [13] Q. Chu, T. Xu, miR-192 targeting IL-1RI regulates the immune response in miiuy croaker after pathogen infection in vitro and in vivo, Fish Shellfish Immunol. 54 (2016) 537–543, <https://doi.org/10.1016/j.fsi.2016.05.007>.
 - [14] K. Eslamloo, S.M. Inkpen, M.L. Rise, R. Andreassen, Discovery of microRNAs associated with the antiviral immune response of Atlantic cod macrophages, Mol. Immunol. 93 (2018) 152–161, <https://doi.org/10.1016/j.molimm.2017.11.015>.
 - [15] M. Wang, S. Jiang, W. Wu, F. Yu, W. Chang, P. Li, K. Wang, Non-coding RNAs function as immune regulators in teleost fish, Front. Immunol. 9 (2018) 1–15, <https://doi.org/10.3389/fimmu.2018.02801>.
 - [16] H.G. Abo-Al-El, The emerging regulatory roles of noncoding RNAs in immune function of fish: MicroRNAs versus long noncoding RNAs, Mol. Genet. Genom. 296 (2021) 765–781, <https://doi.org/10.1007/s00438-021-01786-x>.
 - [17] N.A. Cabillon, C.C. Lazado, Mucosal Barrier Functions of Fish under Changing Environmental Conditions (2019) 1–10, <https://doi.org/10.3390/fishes4010002>.
 - [18] C.M. Press, Ø. Evensen, The morphology of the immune system in teleost fishes, Fish Shellfish Immunol. 9 (1999) 309–318.
 - [19] I. Fernández, J.M.O. Fernandes, V.P. Roberto, M. Kopp, C. Oliveira, M.F. Riesco, J. Dias, C.J. Cox, M. Leonor Canela, E. Cabrita, P. Gavaia, Circulating small non-coding RNAs provide new insights into vitamin K nutrition and reproductive physiology in teleost fish, Biochim. Biophys. Acta Gen. Subj. 1863 (2019) 39–51, <https://doi.org/10.1016/j.bbagen.2018.09.017>.
 - [20] E. Cardona, L. Milhade, A. Pourtau, S. Panerat, F. Terrier, A. Lanuque, J. Roy, L. Marandel, J. Bohe, S. Skiba-Cassy, Tissue origin of circulating microRNAs and their response to nutritional and environmental stress in rainbow trout (*Oncorhynchus mykiss*, Sci. Total Environ. 853 (2022), <https://doi.org/10.1016/j.scitotenv.2022.158584>.
 - [21] F.J. Toledo-Solis, A.M. Larrán, J.B. Ortiz-Delgado, C. Sarasquete, J. Dias, S. Morais, I. Fernández, Specific blood plasma circulating miRNAs are associated with the physiological impact of total fish meal replacement with soybean meal in diets for rainbow trout (*Oncorhynchus mykiss*), Biology 12 (2023), <https://doi.org/10.3390/biology12070937>.
 - [22] C. Houdelet, E. Blondeau, B. Mathilde, E. Villar, X. Mialhe, S. Hermet, F. Ruelle, G. Dutto, A. Bajek, J. Bohe, B. Geffroy, Circulating MicroRNAs indicative of sex and stress in the European seabass (*Dicentrarchus labrax*): toward the identification of new biomarkers, Mar. Biotechnol. 25 (2023) 749–762, <https://doi.org/10.1007/s10126-023-10237-0>.
 - [23] K. Elekkis, C. Papanephytous, The circulating biomarkers league: Combining miRNAs with cell-free DNAs and proteins, Int. J. Mol. Sci. 25 (2024) 3403, <https://doi.org/10.3390/ijms25063403>.
 - [24] S.C. Forster, M.D. Tate, P.J. Hertzog, MicroRNA as type I interferon-regulated transcripts and modulators of the innate immune response, Front. Immunol. (2015), <https://doi.org/10.3389/fimmu.2015.00334>.
 - [25] D.B. Bela-ong, B.D. Schyth, J. Zou, C.J. Secombes, N. Lorenzen, Involvement of two microRNAs in the early immune response to DNA vaccination against a fish rhabdovirus, Vaccine 33 (2015) 3215–3222, <https://doi.org/10.1016/j.vaccine.2015.04.092>.
 - [26] B.C. Zhang, Z.J. Zhou, L. Sun, Pol-miR-731, a teleost miRNA upregulated by megalocytivirus, negatively regulates virus-induced type I interferon response, apoptosis, and cell cycle arrest, Sci. Rep. 6 (2016) 1–14, <https://doi.org/10.1038/srep28354>.
 - [27] M. Smyrli, A. Prapas, G. Rigos, C. Kokkari, M. Pavlidis, P. Katharios, *Aeromonas Veronii* in European Seabass Materials and Methods, vol. 52, 2017, pp. 68–81.
 - [28] M. Smyrli, A. Triga, N. Dourala, P. Varvarigos, M. Pavlidis, V.H. Quoc, P. Katharios, Comparative study on a novel pathogen of european seabass. Diversity of *Aeromonas veronii* in the aegae sea, Microorganisms 7 (2019), <https://doi.org/10.3390/microorganisms7110504>.
 - [29] Anonymous, Guidelines for the treatment of animals in behavioural research and teaching, Anim. Behav. 55 (1998). Animal protection/Animal use.
 - [30] EU, Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes, Off. J. Eur. Union L 276/33 (2010). Animal protection.
 - [31] M. Smyrli, I.Z. Anka, O. Koutsouli, E. Dotsika, I.D. Kyriazis, M. Pavlidis, P. Katharios, Development of autogenous vaccines for farmed European seabass against *Aeromonas veronii* using zebrafish as a model for efficacy assessment, Fish Shellfish Immunol. 123 (2022) 381–387, <https://doi.org/10.1016/j.fsi.2022.03.019>.
 - [32] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data, Bioinformatics 30 (2014) 2114–2120, <https://doi.org/10.1093/bioinformatics/btu170>.
 - [33] S. Griffiths-Jones, H.K. Saini, S. Van Dongen, A.J. Enright, miRBase: tools for microRNA genomics, Nucleic Acids Res. (2008), <https://doi.org/10.1093/nar/gkm952>.
 - [34] F.C. Jones, M.G. Grabherr, Y.F. Chan, P. Russell, E. Mauceli, J. Johnson, R. Swofford, M. Pirun, M.C. Zody, S. White, E. Birney, S. Searle, J. Schmutz, J. Grimwood, M.C. Dickson, R.M. Myers, C.T. Miller, B.R. Summers, A.K. Knecht, S. D. Brady, H. Zhang, A.A. Pollen, T. Howes, C. Amemiya, J. Baldwin, T. Bloom, D. B. Jaffe, R. Nicol, J. Wilkinson, E.S. Lander, F. Di Palma, K. Lindblad-Toh, D. M. Kingsley, The genomic basis of adaptive evolution in threespine sticklebacks, Nature 484 (2012) 55–61, <https://doi.org/10.1038/nature10944>.
 - [35] R. Guyon, F. Senger, M. Rakotomanga, N. Sadequi, F.A.M. Volckaert, C. Hitte, F. Galibert, Genomics A radiation hybrid map of the European sea bass (*Dicentrarchus labrax*) based on 1581 markers : synteny analysis with model fi sh genomes, Genomics 96 (2010) 228–238, <https://doi.org/10.1016/j.ygeno.2010.07.007>.
 - [36] H. Varet, L. Brillet-Guéguen, J.Y. Coppée, M.A. Dillies, SARTools: a DESeq2- and edgeR-based R pipeline for comprehensive differential analysis of RNA-Seq data, PLoS One 11 (2016) 1–8, <https://doi.org/10.1371/journal.pone.0157022>.
 - [37] J. Krüger, M. Rehmsmeier, RNAhybrid: MicroRNA target prediction easy, fast and flexible, Nucleic Acids Res. 34 (2006) W451–W454, <https://doi.org/10.1093/nar/gkl243>.
 - [38] V. Agarwal, G.W. Bell, J.W. Nam, D.P. Bartel, Predicting effective microRNA target sites in mammalian mRNAs, Elife 4 (2015) 1–38, <https://doi.org/10.7554/eLife.05005>.
 - [39] M. Iborra, F. Bernuzzi, P. Invernizzi, S. Danese, MicroRNAs in autoimmunity and inflammatory bowel disease: crucial regulators in immune response, Autoimmun. Rev. (2012), <https://doi.org/10.1016/j.autrev.2010.07.002>.
 - [40] K. Chandan, M. Gupta, M. Sarwat, Role of host and pathogen-derived MicroRNAs in immune regulation during infectious and inflammatory diseases, Front. Immunol. 10 (2020) 1–14, <https://doi.org/10.3389/fimmu.2019.03081>.
 - [41] S.M. Corley, N.M. Troy, A. Bosco, M.R. Wilkins, QuantSeq. 3' Sequencing combined with Salmon provides a fast, reliable approach for high throughput RNA expression analysis, Sci. Rep. 9 (2019) 1–15, <https://doi.org/10.1038/s41598-019-55434-x>.
 - [42] J.D. Campbell, G. Liu, L. Luo, J. Xiao, J. Gerrein, B. Juan-Guardela, J. Tedrow, Y. O. Alekseyev, I.V. Yang, M. Correll, M. Geraci, J. Quackenbush, F. Sciruba, D. A. Schwartz, N. Kaminski, W.E. Johnson, S. Monti, A. Spira, J. Beane, M. E. Lenburg, Assessment of microRNA differential expression and detection in multiplexed small RNA sequencing data, RNA 21 (2015) 164–171, <https://doi.org/10.1261/rna.046060.114>.
 - [43] Z. Sun, J. Evans, A. Bhagwate, S. Middha, M. Bockol, H. Yan, J.P. Kocher, CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data, BMC Genom. 15 (2014) 423, <https://doi.org/10.1186/1471-2164-15-423>.
 - [44] S. Maekawa, P.C. Wang, S.C. Chen, Differential expression genes of the head kidney and spleen in *Streptococcus iniae*-infected East Asian fourfinger threadfin fish (*Eleutheronema tetradactylum*), Int. J. Mol. Sci. 24 (2023) 1–14, <https://doi.org/10.3390/ijms24043832>.
 - [45] C. Bianco, I. Mohr, Ribosome biogenesis restricts innate immune responses to virus infection and DNA, Elife 8 (2019) 1–21, <https://doi.org/10.7554/eLife.49551>.
 - [46] L.E. Fuhrman, A.K. Goel, J. Smith, K. V. Shianna, A. Aballay, Nuclear proteins suppress *Caenorhabditis elegans* innate immunity by inhibiting p53/CEP-1, 5, <https://doi.org/10.1371/journal.pgen.1000657>, 2009.
 - [47] V. Kumar, Introduction to ribosome factory, origin, and evolution of translation, Emerg. Concepts Ribosome Struct. Biog. Funct. (2021) 1–13, <https://doi.org/10.1016/B978-0-12-816364-1.00002-0>.
 - [48] M.-S. Dai, H. Lu, Crosstalk between c-Myc and ribosome in ribosomal biogenesis and cancer, J. Cell. Biochem. 105 (2008) 670–677, <https://doi.org/10.1002/jcb.21895.Crosstalk>.
 - [49] M. Dai, H. Arnold, R. Sears, H. Lu, protein L11 26 (2007) 3332–3345, <https://doi.org/10.1038/sj.emboj.7601776>.
 - [50] S.V. Gnanasundaram, R. Fähræus, Translation stress regulates ribosome synthesis and cell proliferation, Int. J. Mol. Sci. 19 (2018), <https://doi.org/10.3390/ijms19123757>.
 - [51] F. Fontenla, V. Blanco-Abad, B.G. Pardo, I. Folgueira, M. Noia, A. Gómez-Tato, P. Martínez, J.M. Leiro, J. Lamas, Vaccine-induced modulation of gene expression in turbot peritoneal cells. A microarray approach, Mol. Immunol. 75 (2016) 188–199, <https://doi.org/10.1016/j.molimm.2016.06.001>.
 - [52] M. Noia, B. Domínguez, J. Leiro, J. Blanco-Méndez, A. Luzardo-Álvarez, J. Lamas, Inflammatory responses and side effects generated by several adjuvant-containing vaccines in turbot, Fish Shellfish Immunol. 38 (2014) 244–254, <https://doi.org/10.1016/j.fsi.2014.03.020>.
 - [53] S. Mostowy, A.R. Shenoy, The cytoskeleton in cell-autonomous immunity: structural determinants of host defence, Nat. Rev. Immunol. 15 (2015) 559–573, <https://doi.org/10.1038/nri3877>.
 - [54] R. Castro, L. Jouneau, L. Tacchi, D.J. Macqueen, A. Alzaid, C.J. Secombes, S.A. M. Martin, P. Boudinot, Disparate developmental patterns of immune responses to bacterial and viral infections in fish, Sci. Rep. 5 (2015) 1–13, <https://doi.org/10.1038/srep15458>.
 - [55] K.L. Wang, W. Ji, G.R. Zhang, K.J. Wei, Z.C. Shi, X.T. Zhang, H. Zheng, Q.X. Fan, Molecular characterization and expression analysis of three TLR genes in yellow catfish (*Pelteobagrus fulvidraco*): responses to stimulation of *Aeromonas hydrophila* and TLR ligands, Fish Shellfish Immunol. 66 (2017) 466–479, <https://doi.org/10.1016/j.fsi.2017.05.056>.
 - [56] O.M. Delmonte, J.R.E. Bergerson, T. Kawai, H.S. Kuehn, D.H. McDermott, I. Cortese, M.T. Zimmermann, A.K. Dobbs, M. Bosticardo, D. Fink, S. Majumdar, B. Palterer, F. Pala, N.R. Dsouza, M. Pouzolles, N. Taylor, K.R. Calvo, S.R. Daley, D. Velez, A. Agharahimi, K. Myint-Hpu, L.K. Dropulic, J.J. Lyons, S.M. Holland, A. F. Freeman, R. Ghosh, M.B. Similuk, J.E. Niemela, J. Stoddard, D.B. Kuhns, R. Urrutia, S.D. Rosenzweig, M.A. Walkiewicz, P.M. Murphy, L.D. Notarangelo, SARS3 variants cause a novel form of X-linked combined immunodeficiency with immune dysregulation, Blood 138 (2021) 1019–1033, <https://doi.org/10.1182/blood.202008629>.

- [57] Q. Wang, X. Zhao, Y. Liu, J. Zheng, H. Cui, H. Wang, H. Ding, H. Liu, Z. Ding, Characterization and expression analysis of genes from *Megalobrama amblycephala* encoding hemoglobins with extracellular microbicidal activity, *Genes* 14 (2023), <https://doi.org/10.3390/genes14101972>.
- [58] J. Han, G. Xu, T. Xu, The miuiy croaker microRNA transcriptome and microRNA regulation of RIG-I like receptor signaling pathway after poly(I: C) Stimulation, *Fish Shellfish Immunol.* 54 (2016) 419–426, <https://doi.org/10.1016/j.fsi.2016.04.126>.
- [59] J.S. Kwak, M.S. Kim, K.H. Kim, Generation of microRNA-30e-producing recombinant viral hemorrhagic septicemia virus (VHSV) and its effect on in vitro immune responses, *Fish Shellfish Immunol.* 94 (2019) 381–388, <https://doi.org/10.1016/j.fsi.2019.09.026>.