New species for EU aquaculture

Deliverable Report

Deliverable No:	D24.13	**Delivery Month:**	54
Deliverable Title	Description of Immune Gene Expression Pre- and Post-immunization of Meagre		
WP No:	24	**WP Lead beneficiary:**	P1. HCMR
WP Title:	Fish health - meagre		
Task No:	24.4.2	**Task Lead beneficiary:**	P5. UNIABDN
Task Title:	Monitor specific immune responses		
Other beneficiaries:	P3. IRTA		
Status:	Delivered	**Expected month:**	54
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Objective: To gain a better understanding of the immune response in meagre pre- and post- pathogen exposure, immune markers developed in D24.3 will be used to monitor some key innate and adaptive immune responses. Samples collected post-vaccination and post-inoculation with *Vibrio anguillarum* will aid in understanding the effectiveness of vaccination in stimulating an immune response.

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1. Introduction

The objective of this deliverable is to document the immune response of specific genes under conditions of vaccination and against pathogens of significance for commercial aquaculture. This work is necessary and relevant since meagre (*Argyrosomus regius*) is a species still relatively new to aquaculture and vaccines licensed for use with other species of fish grown commercially have not been specifically trialed using *A. regius*. This DL has focused on *Vibrio anguillarum* as it is a pathogen with broad host-range and likely to be of concern for intensive rearing facilities of meagre. The original bacterium for this study was a *Nocardia* species that was hypothesized to be relevant for the systemic granulomatosis that is prevalent among cultured meagre, but as the association of *Nocardia* to this disease has not been proven during the progress of this project we have chosen a different pathogen for study. Tissue samples were collected from fish following vaccination and again after exposure to *V. anguillarum* after which immune gene expression was evaluated for select target genes. Results from this work will be of use to commercial growers and fisheries scientists for understanding in a more detailed manner the specific response of the immune system to commercial vaccine preparations currently available.

2. Materials and Methods

*Materials & Methods: **2017** - Vibrio Challenge*

Most of this is detailed in a previous deliverable, 24.12. Presented below is a brief summary of the materials and methods from the first trial for this experiment. Briefly, following i.p. vaccination of meagre (10g) with an autogenous trivalent Vibrio vaccine (HIPRA), samples of gills, gut, head kidney (HK) and spleen were collected at days 1, 3 and 5 post injection (dpi) for expression analysis of immune genes. Total RNA was extracted from four tissue types and cDNA prepared for qPCR analysis of the following 23 genes:

Innate immunity: Complement factor 3, COX2, lysozyme, metallothionein, NOD2, NOD3, defensin, hepcidin. piscidin

Antiviral genes: IFNc, IFNd, IFNh, Mx

Lymphocyte receptors and associated molecules: IgM. IgT, TcRβ, RAG1

Cytokines: IL-1β, IL-4/13, IL-10, IL-17A/F, IL-22, TNFa1, TNFa2

The data were expressed as arbitrary units relative to the house-keeping gene GAPDH. Data were analyzed for statistical differences between vaccinated fish and fish injected with an equal volume of saline for each day post-injection. Just prior to challenge with live inoculum of *Vibrio anguillarum*, all fish succumbed to a massive ciliated protozoan infection and the post inoculation study was not completed for this year.

*Materials & Methods: **2018** - Vibrio Challenge*

Fish Culture and Experimental Challenge

Juvenile meagre (n= 3000; mean weight= 5.4g) were provided by Alevines del Sureste (Andromeda Group) in Murcia, and transported by road to IRTA (P3) facilities in early May, 2018. Immediately on arrival low numbers of mortalities were observed and fish health screening was performed. All fish were given 3 consecutive prophylactic formalin baths 3 days apart after the observed mortalities were recorded and afterwards held in 1000 L circular tanks to acclimatize to the ambient water conditions of the facilities. Once

acclimatized, again 10 fish were selected for examination of ciliate infestation and kidney biopsies. Briefly, a branchial arch from each fish was removed and mounted on a slide together with scrapings from the lateral skin of the fish, mounted under a coverslip, for observation at 40X magnification to look for accumulations of bacteria, any signs of gill damage or parasites. The body of the animal was then cleaned with alcohol, opened aseptically with sterile scissors and forceps to examine the viscera and collect a kidney biopsy that was used for inoculating onto solid agar culture media (TSA and TCBS). Agar plates were incubated at 23ºC for 24 and 48 hours to check for bacterial growth.

By June when fish had reached a mean weight of 11.5 gm, they were given a bath vaccination of a trivalent vaccine against *Vibrio anguillarum*, *Vibrio harveyi* and *Photobacterium damsela* (Acuipharma, lot# 5.22/17) administered following the manufacturer's instructions. Briefly, meagre (n= 250) were immersed in a vaccine suspension diluted 1:10 with ambient water in groups of 60 for one minute per group. Control fish (n= 250) were treated in a likewise manner using ambient water as a mock vaccination to provide similar handling-stress treatment. After four weeks, the fish were given a booster in a similar manner. Following the booster, the fish were held for another three weeks before challenge with injections of live bacteria near the end of July. Biometry of the fish 5 days prior to challenge found the mean weight of fish was 32.57 gm.

For the bacterial challenge, a strain of *V. anguillarum* previously established as being virulent for meagre (strain IRTA 15-1 provided by HIPRA and used with their permission) was recovered from culture stocks frozen at -80ºC. To estimate the LD_{50} dosage of *V. anguillarum,* a bacterial suspension was prepared from 24 hour cultures grown on TSA media. The bacterial suspension was prepared from colonies that were scraped from the solid media using a sterile bacterial inoculating loop and placed in a tube of sterile PBS, then vigorously vortexed to obtain a homogeneous suspension. The optical density of the suspension was measured using a wavelength of 550 nm on a turbidometer/colorimeter (Dinko Instruments, D-100) and the density of suspension was adjusted until an OD_{550} of ~0.6 was obtained (0.598). Serial 10-fold dilutions of this suspension were prepared in sterile PBS and 100 uL of each suspension spread onto TSA plates and incubated overnight. Colony counts from three replicate platings of each dilution provided the actual number of colony forming units (CFUs) in each suspension that had been injected into fish the previous day. Dilutions of 10^8, 10^7, 10^6, 10^5, and 10^4 were used for injecting juvenile meagre of ~11.5 gms.

For the challenge, one half of the vaccinated fish and one half of the PBS-treated fish were injected with *V. anguillarum* resulting in four treatment groups (**Fig. A**). Meagre were injected intraperitoneally with 100µl of either PBS as a control, or with *V. anguillarum* at a dose of 3.3 X 10^8 CFU/mL (OD_{550}= 0.621). Injected fish were distributed into 100 L tanks connected to a recirculation module (IRTAmar) (n= 15 fish /tank) (**Fig. B**). Ambient water conditions were 7.4 mg/L dissolved oxygen and a temperature of 21.4 ºC. Fish were fed twice daily with Biomar Intro Plus MT (1.5 mm pellet size) and Intro Plus MT (1.9 mm pellet size) when first they were installed at IRTA facilities. As the juveniles increased in size the food was switched to Skretting Optibream L2 (2mm pellet size) until the end of the experiment. Once the meagre had been injected with *V. anguillarum*, all tanks were inspected four times daily at 3 hour intervals to collect any dead fish. Differential mortality data was collected for evaluating the efficacy of vaccination with the trivalent vaccine (Acuipharma). During the first two days post-challenge all fish in one replicate tank (#22) of the vaccinated challenged fish (VAC-VB) died due to hypoxia caused by a plumbing problem. Therefore, one replicate tank was removed from all treatment groups.

At the end of the experiment, necropsies were performed on 6 fish from each Vibrio-injected treatment group to check for carrier status of the remaining fish. Kidney smears inoculated onto TSA (2.5% NaCl) solid media were incubated at 23ºC for 48 hours. Colonies were screened using *V. anguillarum*-specific PCR (Hong et al. 2007).

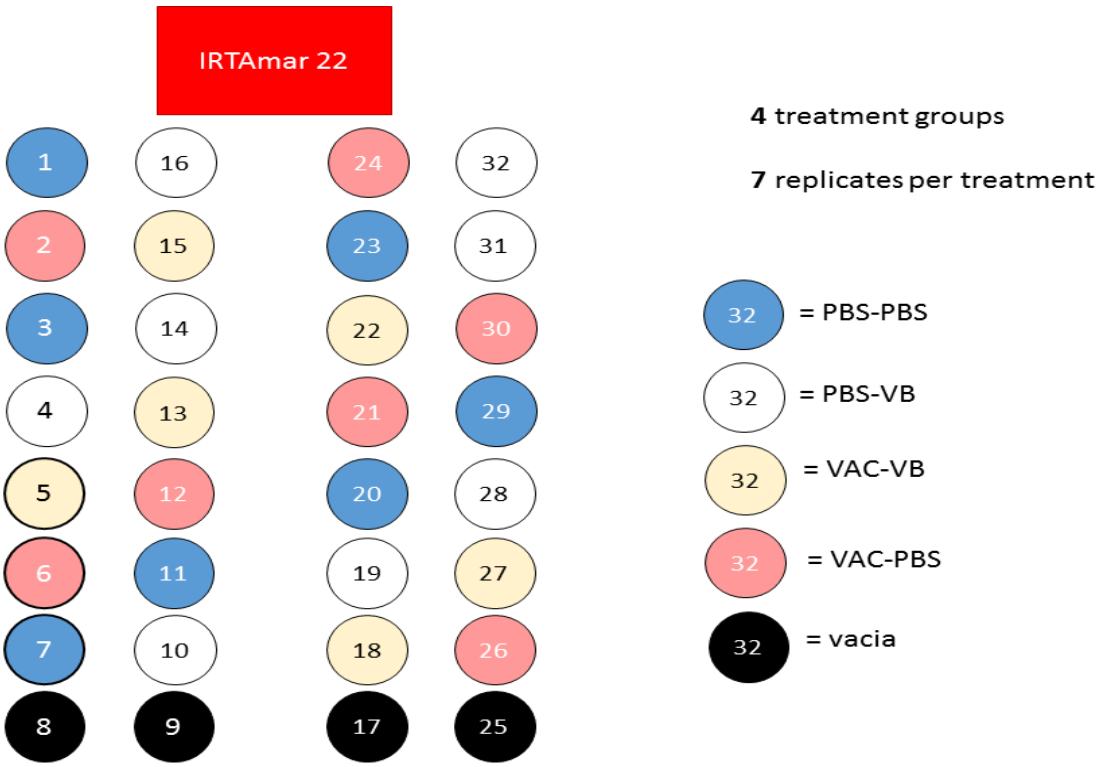


Figure A. Schematic of tank arrangement for different treatments in RAS module. Abbreviations: PBS-PBS= mock vaccination with PBS and mock challenge with PBS; PBS-VB= mock vaccination with PBS followed by injection with *V. anguillarum*; VAC-VB= bath vaccination against *V. anguillarum* followed by injection with *V. anguillarum*; VAC-PBS= bath vaccination against *V. anguillarum* followed by injection with PBS. Tank numbers are shown inside circles.

Identification of Bacterial Contamination

From each TSA (2.5% NaCl) plate corresponding to different fish, a bacterial colony was collected from the agar media using sterile toothpicks. Five isolates were purified by re-streaking on individual new TSA plates. A single colony from these new plates was used for preparing genomic DNA to be used for 16S rDNA PCR. Primers used for 16S rDNA amplifications were previously described (Suzuki et al., 1996). Amplified products were purified (PCR Purification Kit, Qiagen) and the purified products sent for sequencing (Sistemas Genomicos, Valencia, Spain) to obtain approximately 500 bp of unambiguous sequence. The sequence obtained was compared to the database of NCBI using the BLAST utility.

Figure B. Experimental set up using IRTAmar® RAS for meagre challenge with *Vibrio anguillarum*.

Sample Collection

To prepare the fish for sample collections (tissue, blood and mucus) they were euthanized by immersion in ambient water containing an overdose of tricaine methanesulfonate (MS-222, Sigma-Aldrich). Tissue samples (gills, mid-gut, head kidney and spleen) were collected before vaccination, just prior to bacterial challenge, and at 24 and 48 hours after bacterial injections were administered. In addition, baseline samples of blood and skin mucus were collected prior to vaccination and three weeks after the booster (prior to *Vibrio* injection). Blood was collected into heparinized tubes and the serum was separated by centrifugation at 5000 rpm for 10 min and 4ºC, then transferred to clean 1.5 mL Eppendorf tubes and frozen at -20ºC. Mucus was collected by gently scraping the flank of the fish with a plastic spatula and collecting the mucus in a clean petri dish; afterwards this was pipetted into 2 mL Eppendorf tubes and put on ice until later storage in -20ºC. The gills, mid-gut, head kidney and spleen that were dissected were placed immediately in RNAlater and kept at 4ºC for 24 hrs before storage at -80ºC until processing.

Gene Expression analysis

Tissue samples were processed to purify RNA (RNeasy Mini Kit, Qiagen) and the RNA converted to cDNA (Quantitect Reverse Transcription Kit, Qiagen) following manufacturer´s protocols. The cDNA was diluted 1:20 for quantitative comparison of gene expression between pre- and post-vaccination, and challenge samples using the primers shown below in **Table 1** and previously described (Campoverde et al., 2017; Milne et al., 2017; Milne et al., 2018), and reported in D 24.3 and D 24.12. The post-vaccination samples were normalized to baseline samples collected prior to vaccination. The post-challenge samples were normalized to the post-vaccination sample collected just prior to inoculation with live Vibrio culture. The relative expression of all genes was analyzed using GAPDH as an endogenous control. All samples were run in duplicate and each plate included a calibrator sample for inter-plate comparison. Every plate also included a no-template negative control to check for reagent contamination.

Table 1. Primers used for the gene expression analysis of meagre tissue samples.

Target	Name	Sequence	Size (bp)
House-Keeping			
glyceraldehyde phosphate dehydrogenase (sense)	**qpGPDH F**	CCAGTACGTGGTGGAGTCCACTG	109
glyceraldehyde phosphate dehydrogenase (antisense)	**qpGPDH R**	AGCGTCAGCGGTGGGTGCAGAG	
Beta Actin (sense)	**qpBACT F**	TGG GGG AGC AAT GAT CTT GAT CTT CA	212
Beta Actin (antisense)	**qpBACT R**	AGC CCT CTT TCC TCG GTA TGG AGT C	
Target Genes			
A. regius C3 complement (sense)	**qpC3 F**	AACCCATACGCTGTTGCCATGACG	120
A. regius C3 complement (antisense)	**qpC3 R**	CACGTCCTTTAGGTACTGGGCCAG	
A. regius Lysozyme degenerate (sense)	**qpLysoF**	GATGGATCCACTGACTACGGCATC	148
A. regius Lysozyme degenerate (antisense)	**qpLysoR**	ACACGTTTGGCACAGTTGATCGCC	
A. regius NOD 2 (sense)	**qpNOD2 F**	CTCAATACTGTGCTGATGTCCATGG	145
A. regius NOD 2 (antisense)	**qpNOD2 R**	CAAGTGTA**ACCTT**TGGAGT**AAGGT**AG	
A. regius piscicidin (sense)	**qp PISC F**	CAA TGA TCC ATG GGC TTA TCC	111
A. regius piscicidin (antisense)	**qp PISC R**	TTC AGT CTC GCC ATT GAA GC	
A. regius defensin (sense)	**qp DEF F**	GGGAACGAAGATCCAGAGATGCAGTATTGGAC	138
A. regius defensin (antisense)	**qp DEF R**	CTA AGA CCT CAC AGC ACA GCA CCT G	
A. regius hepcidin (sense)	**qp HEPC F**	CCG TCA TGC TCG CCT TCG	140
A. regius hepcidin (antisense)	**qp HEPC R**	CTC ACG CAT GTA ATA CGG AAT CTT GCA TG	
A. regius Ig M (sense)	**qp IGM F**	AAA CTC TAT GAA AGG AGT ATT GGA GGA CA	304
A. regius Ig M (antisense)	**qp IGM R**	CAG ACT CGT GGT GAA CTA CAC AG	
A. regius Ig T (sense)	**qp IgT F**	CCA ACC AAG TCC CAA AGA AA	104
A. regius Ig T (antisense)	**qp IgT R**	GCC ATG TGA TTC TGC TTT CA	
A. regius Interleukin 10 (sense)	**qp IL-10 F**	ACTCCTCGGTCTCTCCTCGTATCCGC	187
A. regius Interleukin 10 (antisense)	**qp IL-10 R**	CTGTGTCGAGATCATCGTTGGCTTCATAAAAGTC	
A. regius Interleukin 1B (sense)	**qp IL-1B F**	GAT TGC CTG GAT TTT CCA CTG TCT CCA	103
A. regius Interleukin 1B (antisense)	**qp IL-1B R**	GTG GCT CTG GGC ATC AAG GG	

2. Results

2017 *- Results post-vaccination*

Prior to challenge with live inoculum of *Vibrio anguillarum* there was a mass epizootic of a ciliated protozoan (*Amiloodinium spp*.) that killed all the fish in the experiment. Thus, there was no actual live bacterial challenge in 2017. Necropsies of some affected fish exhibited adhesions among the mesenteries of the gut that appeared to constrict the bowel in some instances. Absence of feed in such afflicted individuals suggests the lack of feeding may have exacerbated the problem with the protozoan infection.

With regard to gene expression analysis of samples collected pre- and post-vaccination, most of the immune genes there was a clear pattern of modulation post-vaccination. Whilst there were some tissue specific patterns observed, in general most innate genes were up-regulated early post-vaccination, as seen with the proinflammatory cytokines IL-1β and TNFα (**Figs. 1-2**). In some tissues this expression was sustained to day

5 pi, but in others it had returned to control levels by this time (eg.- in gills and head kidney). The antimicrobial peptides were also increased early in many tissues but with a clear difference in expression pattern. For example, defensin was increased at day 1 in gut and spleen, whilst hepcidin was increased at day 1 in gills (**Fig. 3**). Hepcidin expression was also high in vaccinated fish at days 3 and 5, presumably as a consequence of PAMP stimulation by the vaccine components. The antiviral genes showed no clear trends, although by day 5 pi, Mx expression was significantly inhibited in the vaccinated fish, especially in the systemic tissues (head kidney and spleen). This may be a consequence of the antibacterial pathways being activated, rather than the interferon pathway following bacterial vaccination. Of the genes monitored that are associated with adaptive immunity (Igs, TcR, Th cytokines), IgM and IgT expression both increased at day 3 and/or day 5 post-vaccination (**Figs. 4-5**), the latter only in gills and gut, in line with its function as a mucosal antibody. In contrast, TcR expression was not modulated. Of the cytokine genes examined that are considered to be T-cell derived, IL-4/13, IL-10 and IL-17A/F showed little if any modulation. However, IL-22 was increased, again at the later time points (day 3 pi and/or 5 pi) (**Fig. 6**). IL-22 is known to be produced by Th17 cells in mammals, and is linked to induction of defenses to extracellular bacteria, in line with the responses expected after vaccination with a bacterial pathogen (eg.- Vibrio).

Fig. 1.

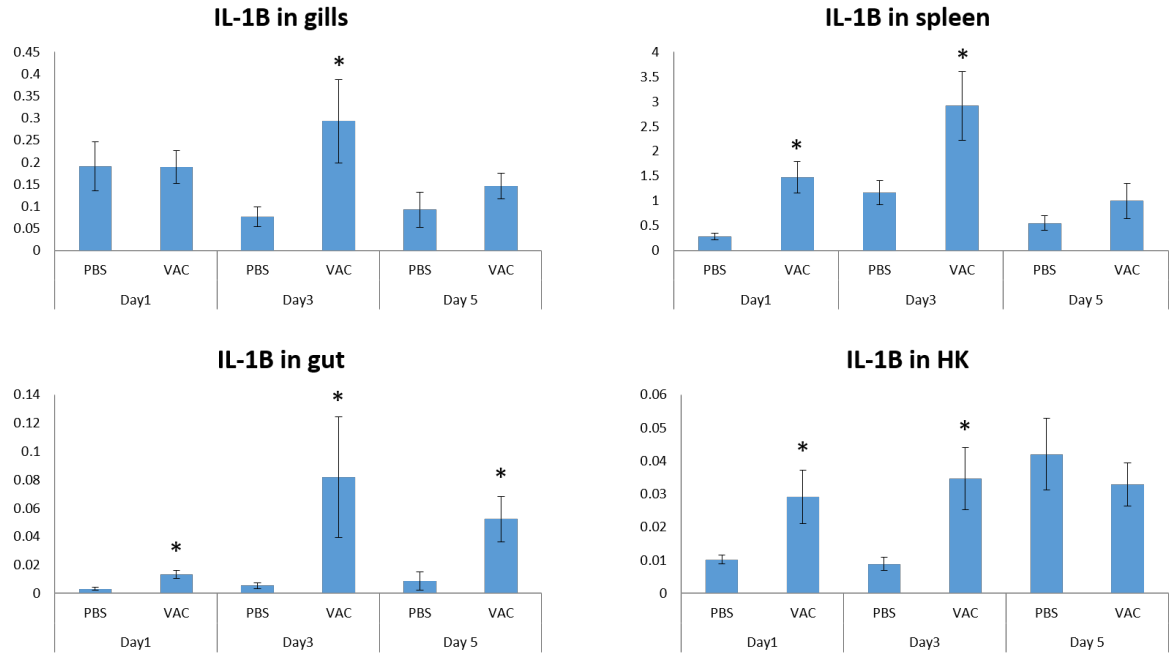


Fig. 2.

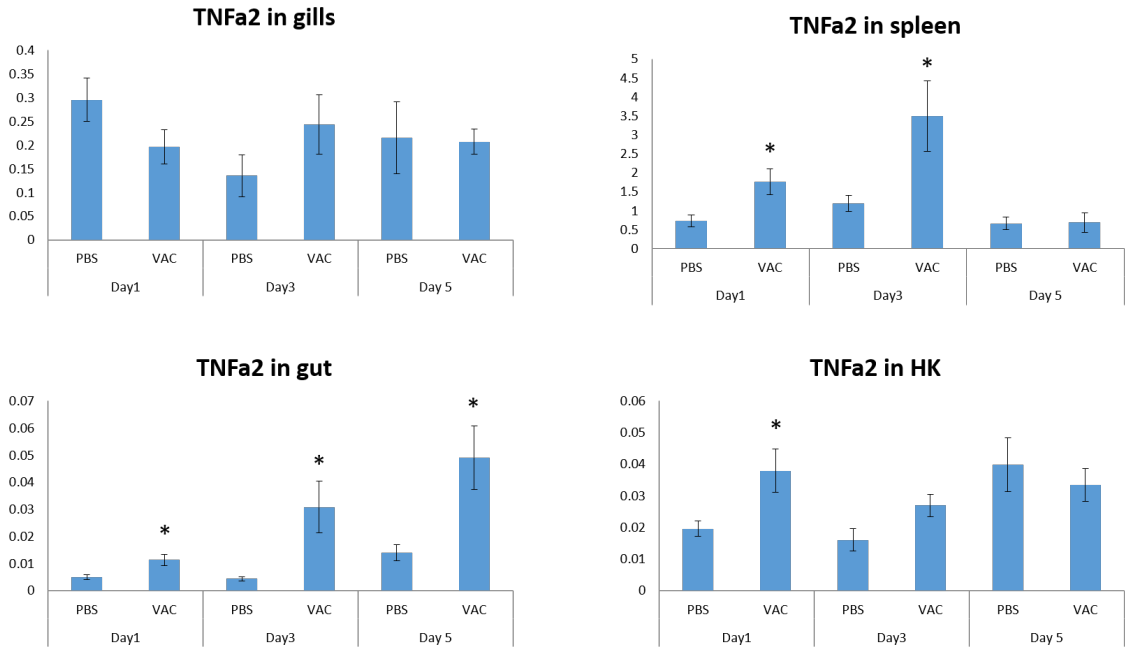

TNFa2 in gills
TNFa2 in spleen
TNFa2 in gut
TNFa2 in HK
PBS
VAC
Day1
Day3
Day 5

Fig. 3.

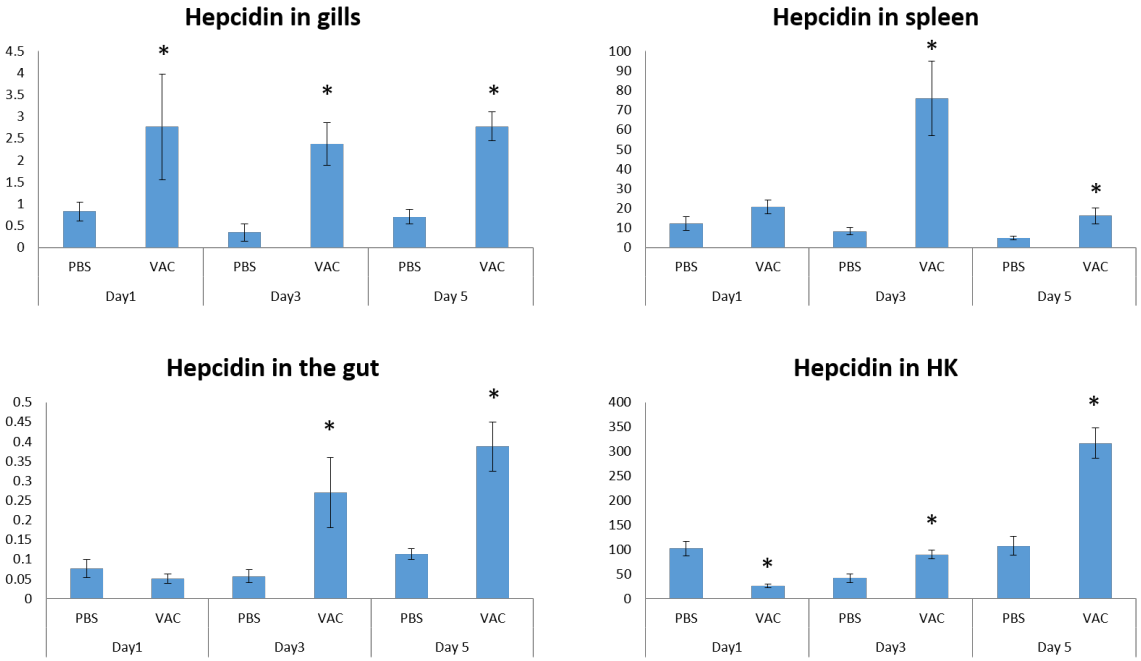

Hepcidin in gills
Hepcidin in spleen
Hepcidin in the gut
Hepcidin in HK
PBS
VAC
Day1
Day3
Day 5

Fig. 4.

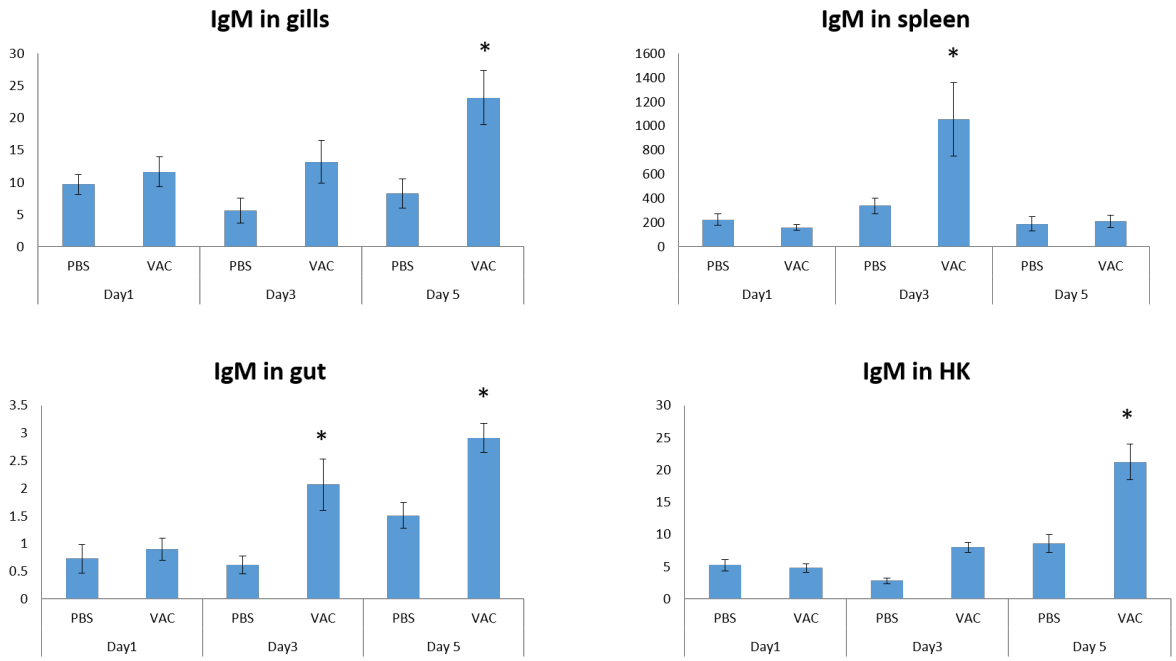

IgM in gills
IgM in spleen
IgM in gut
IgM in HK
PBS
VAC
Day1
Day3
Day 5

Fig. 5.

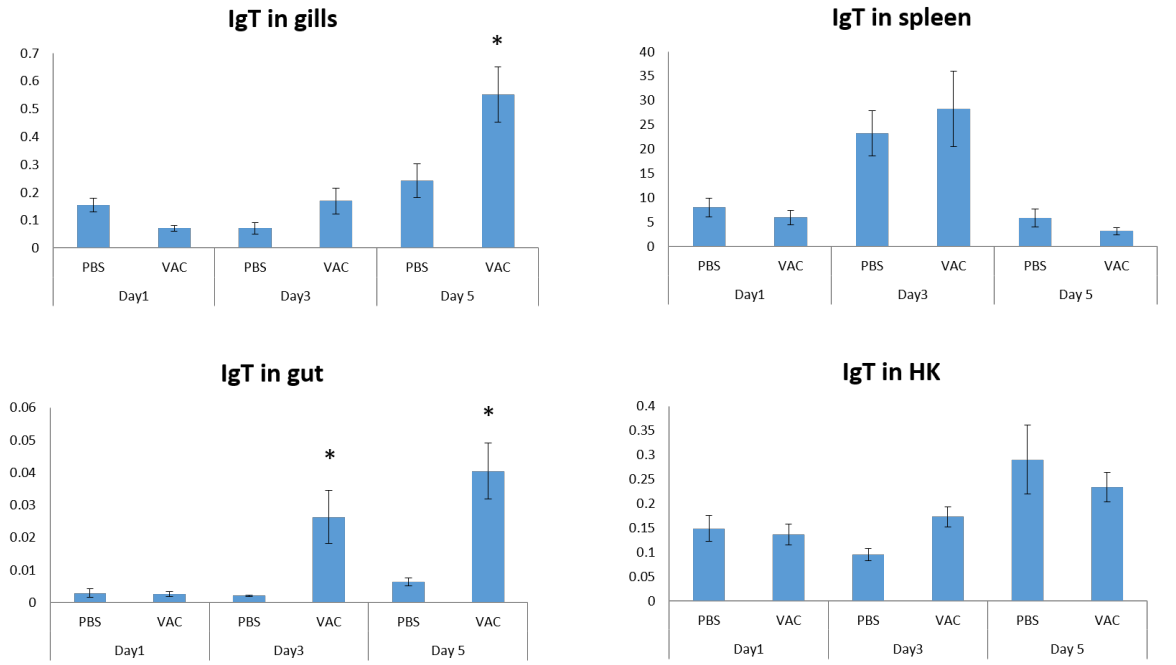
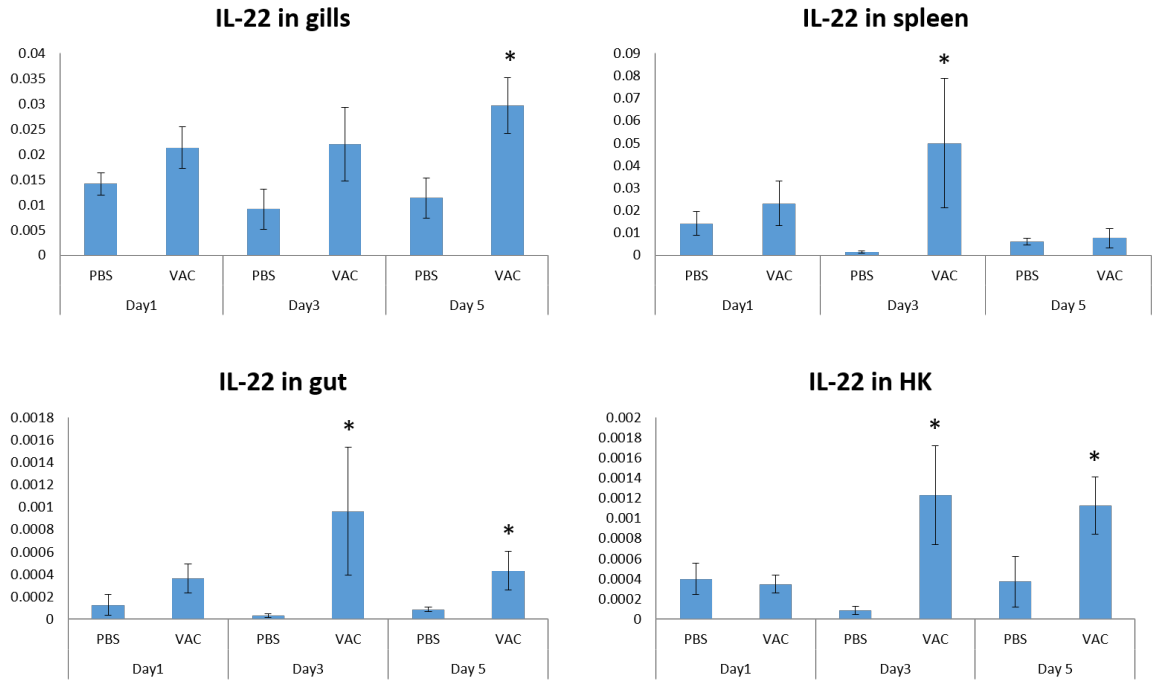

IgT in gills
IgT in spleen
IgT in gut
IgT in HK
PBS
VAC
Day1
Day3
Day 5

Fig. 6.



Figures 1-6. Gene expression from 23 genes analyzed during 2017 in a time-series sampled after vaccination.

2018 *- Establishing Health Status of the Fish Lot Used for Experimentation and Identification of Bacterial Contaminants*

Upon arrival of the meagre juveniles at IRTA facilities, low numbers of mortalities were collected daily (1-4 per day). No external signs of disease were evident, but kidney smears from 5 of 10 (50%) apparently healthy fish that had been plated onto TCBS and TSA agar plates to check for septicemia showed mild growth of a pure culture after 24 hours at 23 ºC. No growth appeared on TCBS media. All of the meagre were given prophylactic baths in formalin to prevent the problems with ciliate infections that occurred the previous year and led to the delay of completion of this deliverable. After the third formalin bath 10 fish were sampled randomly to check their gills and skin scrapings visually by light microscopy, and also to dissect and remove kidney biopsies for spreading onto TSA and TCBS agar plates again. No pathologies were detected in any fish and no bacterial growth was observed on the agar media.

Bacterial 16S rDNA primers were used to amplify ribosomal DNA sequences from the pure cultures obtained from the first fish screening. The obtained sequence was the same from all fish and when analyzed using BLAST was a 99% match to *P. fluorescens* and *Pseudomonas koreensis*. During the acclimatization period (3 weeks) there were no further mortalities. For further details of this aspect of the work see Deliverables D24.6 and D24.12 from this project.

2018 *- Results post-vaccination and post-challenge with Vibrio anguillarum*

Due to time constraints with the project ending in a few weeks, a limited panel of genes, selected from the results of D 24.3, were analyzed using samples collected from the Vibrio challenge experiment conducted in 2018. Mucosal tissues (gill and intestine) and lymphoid tissues (kidney and spleen) were collected as in 2017. However, again due to time constraints only data from the gill tissue is presented in this report. As the gill tissue is in direct and continuous contact with the external environment, and all the potential opportunistic pathogens present, this seemed an appropriate tissue for emphasis in a brief preliminary report. The remaining tissue samples will be processed as time permits after closure of the project.

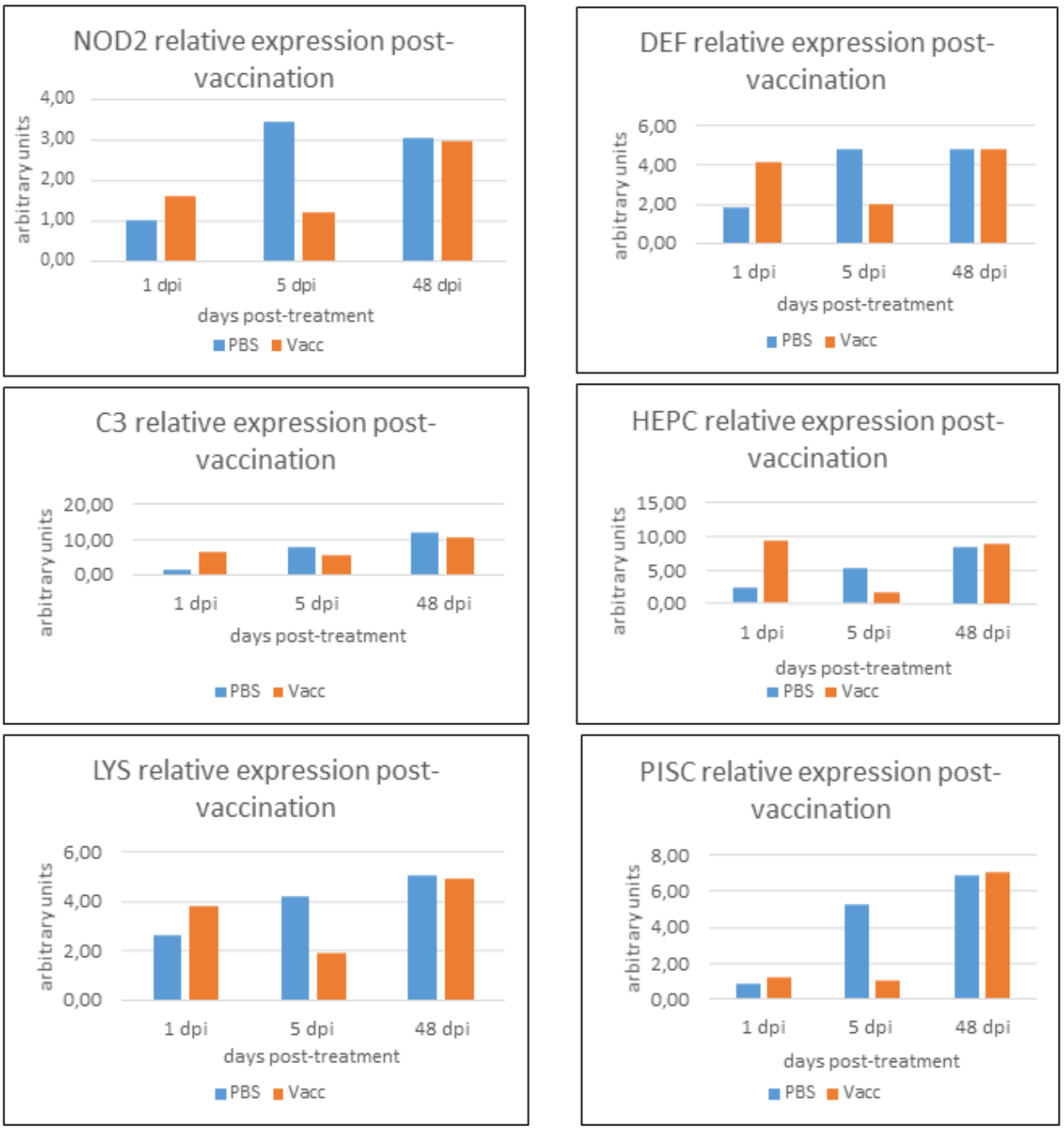


Figure 7. Time series showing changes in expression of antibacterial immune effectors, relative to PBS controls, post-vaccination. Expression is normalized to GAPDH.

Since this was a bacterial challenge, focus was placed on innate genes effecting protection against bacteria and adaptive immune memory response that would provide more lasting protection. The following 10 genes were analyzed.

Antibacterial response: Complement factor 3, lysozyme, NOD2, defensin, hepcidin, piscidin.

Immunoglobulins: IgM. IgT

Cytokines: IL-1β, IL-10

During the time series post-vaccination (1, 5, and 48 dpi) the pattern of expression for all the genes studied followed a similar pattern (**Figs. 7-8**). At 1 dpi the vaccinated samples exhibited an increase in expression relative to the PBS injected control. The maximum value of expression varied among the targeted genes but the result was clear that vaccination increased expression over the contemporary PBS injected controls. At 5 dpi the reverse trend was seen for most genes; the PBS control was somewhat more highly expressed than the vaccinated samples. By 48 dpi the vaccinated and PBS samples were more or less equivocal. Notable are the much higher responses of IL1B and IL-10.

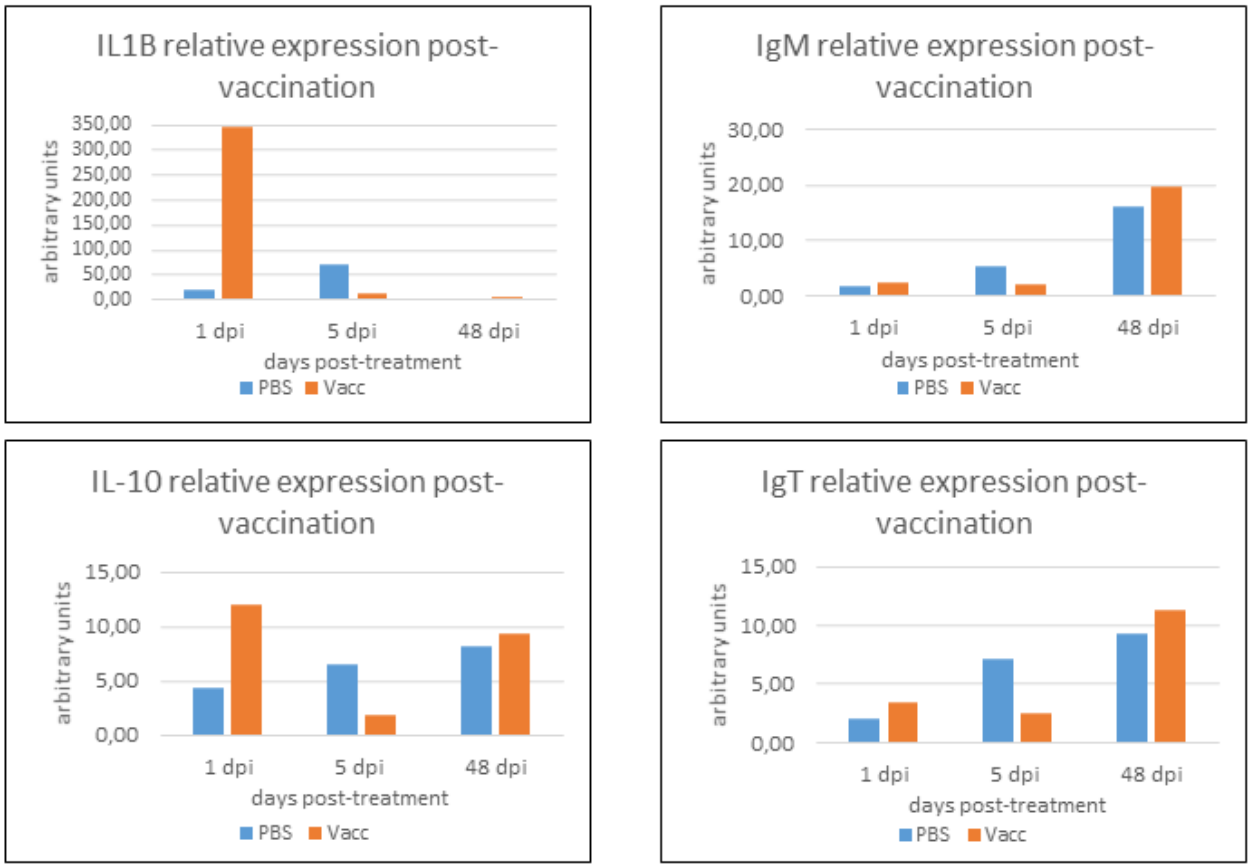


Figure 8. Time series showing changes in expression of adaptive immune response effectors, relative to PBS controls, post-vaccination. Expression is normalized to GAPDH.

After injection with 10^8 CFUs of *Vibrio anguillarum* the expression pattern for each gene varied at each of the time points, with the vaccinated and Vibrio-injected samples exhibiting somewhat different patterns relative to the vaccinated and PBS controls. Much higher levels of expression were exhibited post-

vaccination than after injection with *V. anguillarum*. The control group injected with PBS and then challenged with live bacteria showed higher expression for NOD2, C3, IgM, IL-10, and especially for HEPC and IL1B. The vaccinated fish that were challenged showed the greatest increase in expression for IL1B, HEPC and IgT.

Figure 9. Changes in relative expression, as compared to PBS controls, of antibacterial immune effectors shown for two time points post-vaccination. Expression is normalized to GAPDH.

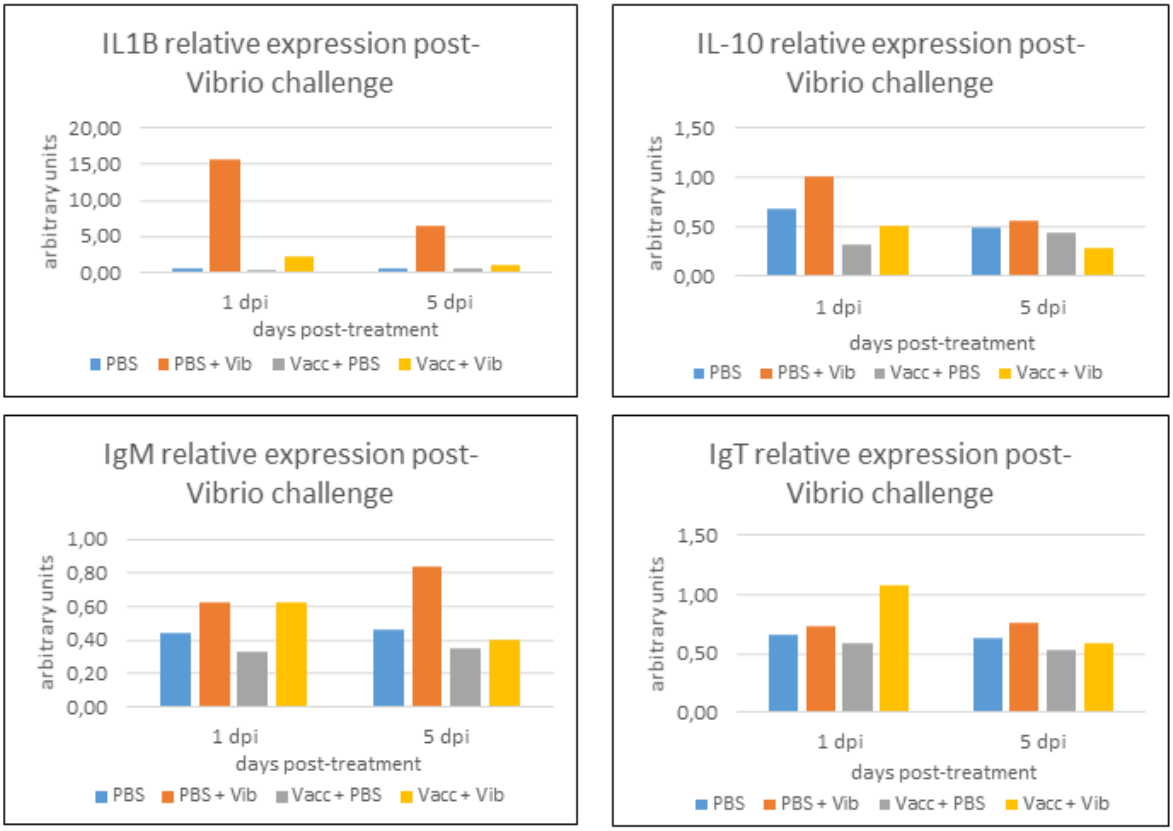


Figure 10. Changes in relative expression, as compared to PBS controls, of adaptive immune response effectors shown for two time points post-vaccination. Expression is normalized to GAPDH.

Biometry was monitored for adjusting feed during the course of the experiment. The mean weight of fish was not equal in each group of treated fish at the end of the experiment (**Table 2**). The standard deviation increased substantially during the course of the experiment.

Cumulative Mortality

No fish from any of the control treatments died during the course of the experiment. The vaccine was effective in stimulating immune memory for providing protective immunity. After six days no further mortalities were observed in any treatment tanks. Total mortality results for all treatment groups is shown below (**Table 3**).

Table 2. Biometry results from the beginning of the trial to the end of trial.

	PBS-PBS		PBS-VIB		VAC-VIB		VAC-PBS	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
June 05	11,53	1,238						
July 01	22,70	5,179						
July 12	32,76	11,237	29,42	9,618	30,02	10,705	32,37	11,219
Aug 03	37,08	9,362	36,16	9,225	33,89	9,759	35,70	9,390

Additional results will be forthcoming with regard to the gene expression in intestine, kidney and spleen samples still to be analyzed. Blood serum and skin mucus samples collected for analysis of the antibody response are still in process, delayed due to problems with growing the bacteria for coating the plates for the ELISA´s. This will be concluded after the end of the project.

Table 3. Total mortality recorded during ten days post-injection using 3.3 X 10^8 CFU/mL of *V. anguillarum*. Mean weight of those that died is shown.

Treatment	Total Mortality	Mean Weight (SD)
PBS-PBS	0%	-
PBS-VIB	17.5%	27.01 (4.8)
VAC-VIB	0%	-
VAC-PBS	0%	-

3. Discussion

From the results of samples collected post-IP injection with vaccine from 2017, it is clear that vaccination by IP injection with this adjuvant-augmented preparation triggers in meagre a defined suite of immune genes, linked to pro-inflammatory and antimicrobial defense. That the Ig and IL-22 responses were somewhat delayed is also interesting and likely linked to the requirement for lymphocyte expansion (clonal proliferation) that typically gives later, but more prolonged protective immunity, compared to innate responses. Whether these responses confers resistance to disease needs to be determined by challenge experiments that were not accomplished in 2017. The infestation of amyloodinium that killed all the fish in 2017 required the work to be repeated in 2018.

The work was completed in 2018, but not without complications since the new stock of fish arrived with a subpopulation of the fish infected with *P. fluorescens* (see D24.12 for details). Holding the fish for three weeks after their arrival and providing them with preventive chemotherapeutic baths of formalin provided the fish with a resting period to recover from transport stress, the likely driver influencing the occurrence of septicemia in a subpopultion of the fish. This elicited some complications later for gene expression analysis (see below). The completed challenge trial did demonstrate the vaccine is effective in providing protective immunity as none of the vaccinated fish died during the trial time course (**Table 3**).

The gene expression analysis from samples collected from the trial conducted in 2018 using a bath vaccine is not yet complete though a challenge with *V. anguillarum* was completed in September 2018. The preliminary data presented herein for the gill tissue samples (**Figs. 7-8**) confirm earlier observations from the analyses conducted in 2017 with the IP injected vaccine. There is an early up-regulation of gene expression from all genes shortly after vaccination (1 dpi), while it subsides shortly after (5 dpi), and later recovers to levels equivalent to the PBS controls (48 dpi). The most robust response was seen from IL1B, which would have downstream consequences for many other immune genes due to this cytokines far-reaching effect.

The high degree of up-regulation of genes post-vaccination like HEPC can be attributed in part to regulation by pro-inflammatory cytokines like IL1B, while the modulatory role of IL-10 (Piazzon et al. 2015) likely explains the low level of expression of PISC. This result of PISC expression under stimulation of live *V. anguillarum* cells is in agreement with results obtained previously (Campoverde et al. 2017) in which HEPC was more highly expressed than PISC in gills under stimulation by LPS injection.

In regard to the Vibrio challenge, the injection with 10^8 CFUs of *V. anguillarum* stimulated a wide variety of response depending on the treatment (vaccinated or non-vaccinated), and the gene being analyzed

(Figs. 9-10). Significance of response was not evident in most cases, but as a trend there were more genes up-regulated (NOD2, C3, HEPC, IL1B, IL-10, IgM) from the PBS control group injected with Vibrio than from the other three treatment groups. The expression of IL1B and HEPC were especially high as compared to the other treatment groups. In the group vaccinated and later injected with *V. anguillarum*, only IL1B, HEPC and IgT were highly expressed. Since IgT is a mucosal antibody this would be expected in the gill tissue as would the expression of HEPC as reported previously (Campoverde et al. 2017). The expected high levels of expression of immune genes from the vaccinated and challenged fish group were not seen. A complicating factor was the fish arriving already contaminated with septicemia caused by *P. fluorescens.* Since not all fish were apparently carriers (~50%) of this mildly virulent pathogen there were fish mixed together in the experimental population with different histories regarding their vaccination status. Some fish had their immune system primed in a non-specific way towards bacterial pathogens, while others were completely naïve immunologically. This means there is a wide range of potential responses due in part to the immunological history of individual fish but also due in part to the size dispersion that manifested itself during the course of the trial (**Table 2**). The differences in size would also imply a difference in physiological maturity among individuals within each treatment cohort. This is something to consider in future such trials.

To conclude, we have evaluated two different vaccine preparations in the trials performed. Both IP injection (2017) and bath vaccination (2018) appear to stimulate positively immune responses of a diverse repertoire. Further, the bath vaccine was demonstrated to provide protective immunity against challenge with *V. anguillarum*. This data (and other results from this project both published and unpublished) shows that the meagre immune response is robust and can be tailored to the potential type of pathogen that is encountered. From the data presented from 2017, it is clear responses vary by tissue, likely linked to the cell composition present at different immune sites. Differences in response from each gene may also reflect a need for immediate or a more prolonged response. These data, and related published work from this project, show the potential to modulate immune responses in meagre in culture, such as by delivery of immuno-stimulants, to enhance particular immune pathways at a time of disease risk such as prior to transport.

5. References

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Deviations

This deliverable has focused on vaccine trials using *Vibrio anguillarum* as a challenge model organism. In the original text of the DoW there was supposed to be used a novel species of Nocardia that was expected as the cause of chronic systemic granulomatosis, but this etiology was not proven during the course of the project and specific deliverables relating to the etiology of systemic granulomatosis. Therefore, it was decided that the focus of the vaccine trials would be *V. anguillarum*. Approval for this change of the DoW was obtained previously.

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