Larvae and Juvenile Lumpfish (*Cyclopterus lumpus*) Immune Response to Oral Immunization Against *Vibrio anguillarum*

by

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ABSTRACT

Vibrio anguillarum is a common marine pathogen that causes the disease vibriosis in several finfish species, including lumpfish (*Cyclopterus lumpus*). The lumpfish is utilized as a cleaner fish to control sea lice in the Atlantic salmon (*Salmo salar*) aquaculture industry in the North Atlantic region. Lumpfish have the ability to visualize and prey upon the ectoparasite sea lice (*Lepeophtheirus salmonis*) found on Atlantic salmon skin. Lumpfish immunity is critical for their optimal performance and sea lice removal as they are able to significantly reduce sea lice infestations up to 93–97% on Atlantic salmon in sea pens.

Oral vaccine delivery at a young age is the desired method for fish immunization. Oral vaccines are easy to use, reduce fish stress during immunization, and can be applied on a large scale while the fish are at a young age. However, the efficacy of orally delivered inactivated vaccines is controversial.

In this study, I evaluated the effectiveness of a bacterin preparation against *V. anguillarum* orally delivered to cultured lumpfish and contrasted it to an intraperitoneal (i.p.) boost delivery. I bio-encapsulated *V. anguillarum* bacterin in *Artemia salina* (live feed) and orally immunized lumpfish larvae. The innate and adaptive immune responses of lumpfish larvae were evaluated by using real-time quantitative polymerase chain reaction (qPCR) analyses. Although the oral *V. anguillarum* vaccine delivered in *A. salina* live feed reached the lumpfish gut, real-time quantitative polymerase chain reaction (qPCR) analyses of immune-relevant transcript expression levels revealed that it only modestly immune-stimulated the lumpfish larvae. Nine months later, lumpfish were either orally, or

orally and i.p boosted with the vaccine and two months later they were challenged with *V. anguillarum* ($7.8x10^5$ CFU $dose^{-1}$). Oral immunization of lumpfish delayed mortality but did not confer protective immunity against the *V. anguillarum* challenge, which is in contrast to the i.p. vaccination which was protective.

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LIST OF ABBREVIATIONS AND SYMBOLS

~	Approximately
°C	Degrees centigrade
BD	Becton Dickinson
CAD	Canadian dollar
ccl19	*C-C motif chemokine-like 19*
ccl20	*C-C motif chemokine-like 20*
cd4	*T-cell surface glycoprotein CD4*
cd4a	*T-cell surface glycoprotein CD4_a*
cd4b	*T-cell surface glycoprotein CD4_b*
cd74	*HLA class II histocompatibility antigen gamma chain*
cd8	*T-cell surface glycoprotein CD8*
cd8a	*T-cell surface glycoprotein CD8 alpha chain*
cDNA	Complementary DNA
CDRF	Cold-Ocean Deep-Sea Research Facility
CFU	Colony forming units
cox2	*Cyclooxygenase-2*
C_T	Cycle threshold
d	Day
DCs	Dendritic cells
DNA	Deoxyribonucleic Acid

Dph	Days post-hatch
DTAF	5-([4,6-dichlorotriazinyl] amino) fluorescein hydrochloride
ef1a	*Elongation factor 1 alpha*
ef1a_a	*Elongation factor 1 alpha_a*
ef1a_c	*Elongation factor 1 alpha_c*
etif3d	*Eukaryotic translation initiation factor 3 subunit D*
FAO	Food and Agriculture Organization
FK J223	Formalin-killed *A. salmonicida* J223 strain
g	Grams
GTP-binding	Guanosine triphosphate binding
h	Hours
hamp	*Hepcidin anti-microbial peptide*
HLA	Human leukocyte antigen
i.p.	Intraperitoneal injection
igh	*Immunoglobulin heavy locus*
igha	*Immunoglobulin heavy chain variable region*
ighb	*Immunoglobulin heavy chain_b*
ighd	*Immunoglobulin delta heavy chain*
ighma	*Immunoglobulin mu heavy chain_a*
ighmc	*Immunoglobulin mu heavy chain_c*
IgM	*Immunoglobulin M*

il10	*Interleukin 10*
il12	*Interleukin 12*
il17	*Interleukin 17*
il1b	*Interleukin 1 beta*
il6	*Interleukin 6*
il8	*Interleukin 8*
il8a	*Interleukin 8_a*
il8b	*Interleukin 8_b*
infg	*Interferon gamma*
IPM	Integrated Pest Management
irf7	*Interferon regulatory factor 7*
IROMPs	Iron-regulated outer membrane proteins
ISAV	Infectious salmon anaemia virus
ISAV NA-HPRΔ	Infectious salmon anaemia virus isolates of North American genotype
ISAV-HPR0	Avirulent infectious salmon anaemia virus isolates of European genotype
IU	International unit
J360	*Vibrio anguillarum* J360 strain
JBARB	Dr. Joe Brown Aquatic Research Building
Kb	Kilobyte

KCl	Potassium chloride
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
l	Litre
lgp2	*ATP-dependent RNA helicase lgp2*
LPS	Lipopolysaccharide
Ly6/uPAR	*The Ly6 (lymphocyte antigen-6)/uPAR (urokinase-type plasminogen activator receptor)*
ly6g6f	*Lymphocyte antigen 6 complex locus protein G6f*
mg	Milligram
MHC	Major histocompatibility complex
min	Minutes
mL	Millilitre
mm	Millimeter
mM	Millimolar
mRNA	Messenger RNA
MUN	Memorial University of Newfoundland
mxa	*Interferon-induced GTP-binding protein_a*
mxb	*Interferon-induced GTP-binding protein_b*
mxc	*Interferon-induced GTP-binding protein_c*
Na_2HPO_4	Disodium hydrogen phosphate

NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
ng	Nanogram
NL	Newfoundland and Labrador
nM	Nanomolar
O.D.	Optical density
pabpc1_a	*Polyadenylate-binding protein 1_a*
pabpc1_b	*Polyadenylate-binding protein 1_b*
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QC	Quality control
qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
rpl32	*60S ribosomal protein L32*
rpm	Revolutions per minute
RPS	Relative Percent Survival
RQ	Respiratory Quotient
rsad2	*Radical S-adenosyl methionine domain containing protein 2 / viperin*
saa5	*Serum amyloid A 5*
SAV-3	Salmonid alphavirus subtype-3

sec	Second
SEM	Standard error of the mean
stat1	*Signal transducer and activator of transcription 1*
subs.	Subspecies
T3SS	Type III Secretion Systems
TLR	Toll-like receptor
tlr3	*Toll-like receptor 3*
tlr5	*Toll-like receptor 5*
tlr5a	*Toll-like receptor 5_a*
tlr5b	*Toll-like receptor 5_b*
tlr7	*Toll-like receptor 7*
tnf	*Tumour necrosis factor*
tnfa	*Tumour necrosis factor alpha*
TOIs	Transcripts of interest
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
U	Unit
UK	United Kingdom
UV	Ultraviolet
Wph	Weeks post-hatch
Wpi	Weeks post-immunization
µg	Microgram

µl	Microlitre
µm	Micrometer

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CO – AUTHORSHIP STATEMENT

The research described in this thesis was designed and conducted by My Dang with guidance from supervisor Dr. Javier Santander and co-supervisor Dr. Jillian Westcott. All experiments, laboratory work, data collection and analysis, and thesis writing was conducted by My Dang, with with assistance from Trung Cao, Ignacio Vasquez, Ahmed Hossain, Hajarooba Gnanagobal, and collaborators Surendra Kumar, Jennifer Monk, and Danny Boyce. Additionally, Dr. Jennifer Hall provided technical support for the quantitative polymerase chain reaction (qPCR) data analyses of this research.

All the chapters were written by My Dang, with suggestions, recommendations and edits provided by Dr. Santander. Additionally, Dr. Westcott provided valuable suggestions in the preparation of the thesis manuscript, as well as the manuscript published in the journal Vaccines (July 23, 2021).

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1. GENERAL INTRODUCTION

1.1. Global Atlantic salmon industry

Globally, farmed Atlantic salmon (*Salmo salar*) is the most cultivated salmonid species, followed by rainbow trout (*Oncorhynchus mykiss*), and coho salmon (*Oncorhynchus kisutch*) (FAO, 2019). The salmonid aquaculture industry has witnessed tremendous growth in production volume and revenues (Asche et al., 2013). According to the Food and Agriculture Organization (FAO; 2020) of the United Nations, worldwide Atlantic salmon production reached 1.434 million tonnes ($9 billion CAD) in 2007 and increased to 2.248 million tonnes in 2016. In 2018, the production continued to increase and reached 2.5 million tonnes, increasing 5–6% over the previous year (Marine Harvest, 2019). Production volumes are expected to increase an additional 4% from 2018 to 2022 (Ernst & Young AS, 2018). Growth of the global Atlantic salmon industry was approximately 180% for the period between 2000 to 2018 (6% compounded annual growth rate), with the range varying between -4% and 22% annually (Marine Harvest, 2019).

The five major Atlantic salmon producing countries, Norway, Chile, the UK, Canada, and the Faroe Islands produced up to 95.6% of total global Atlantic salmon in 2018 (Figure 1-1). Norway produced 55.3%, followed by Chile (25.4 %), UK (7.6%), Canada (6%), and Faroe Islands (3.3%). The remaining countries contributed 4.4% of total production (Iversen et al., 2020).

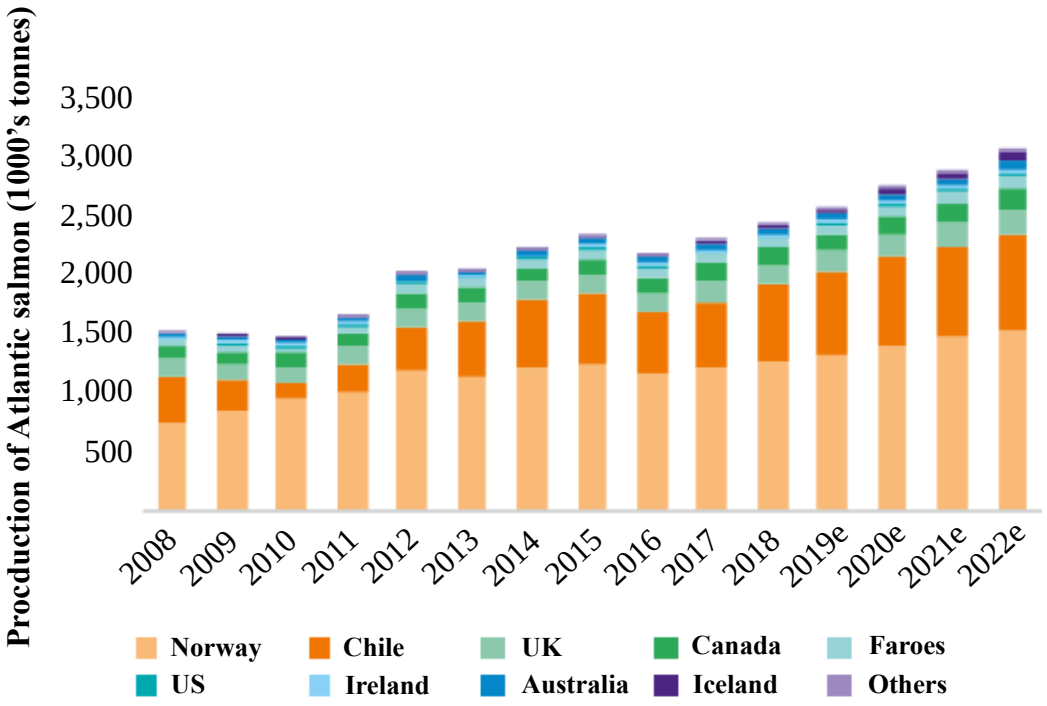

Figure 1-1. The production of Atlantic salmon in the five biggest producer countries from 2008 to 2018 (Iversen et al., 2020).

The Atlantic salmon farming industry significantly contributes to Canadian seafood production (Canadian Aquaculture Industry Alliance, 2018) (Fig. 1-2). In 2010, Canada became the 4th largest producer of farmed Atlantic salmon globally with 101,544 tonnes (DFO, 2013; Sarker et al., 2013). In 2013, more than 100,027 tonnes of Atlantic salmon were produced in Canada with a value of $635,059,000 CAD (DFO, 2013), and by 2014 the yield of salmon decreased to 78,979 tonnes (DFO, 2014). As of 2019, Canada is the fourth-largest producer of farmed Atlantic salmon after Norway, Chile, the UK with a production volume of 118,630 tonnes, corresponding to a market value of $ 914,282,000 CAD (DFO, 2019; Iversen et al., 2020).

British Columbia (BC) leads Canadian farmed finfish production with 95.7% Atlantic salmon, 2.7% Chinook salmon, and 1.6% sablefish (DFO, 2018). British Columbia is the largest farmed Atlantic salmon producer, contributing 92,926 tonnes in 2015 (DFO, 2015). The salmon production volume reached 88,874 tonnes in BC in 2019, valued at $662,749 million (DFO, 2019). New Brunswick was the second-largest producer in Canada in 2019 with 22,395 tonnes of Atlantic salmon production (DFO, 2019).

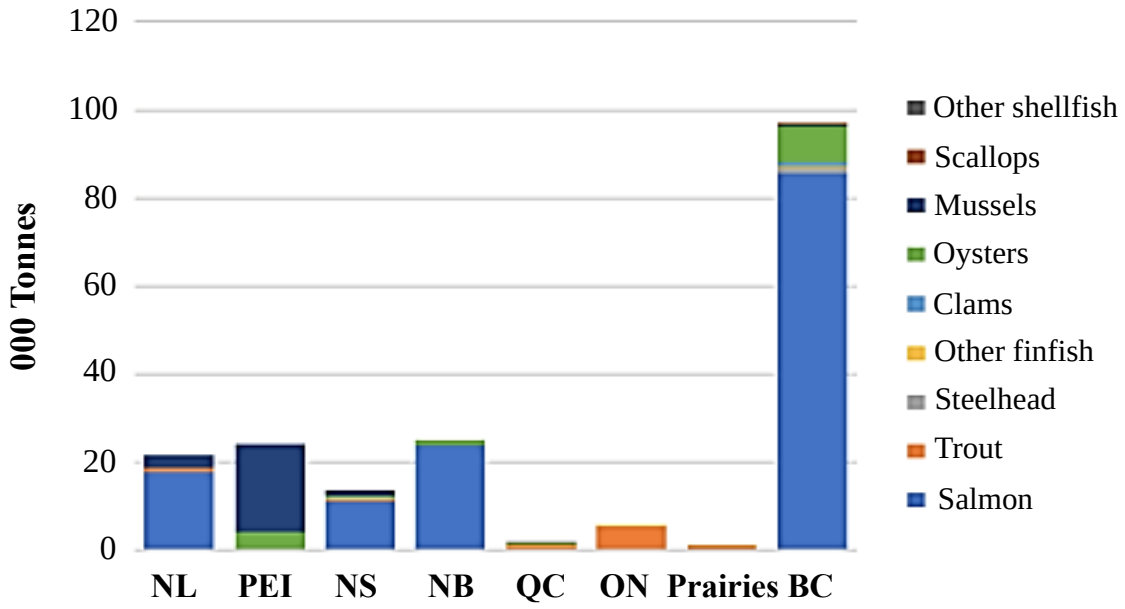

Figure 1-2. Canadian farmed seafood production by province and species in 2017 (Canadian Aquaculture Industry Alliance, 2018)

Atlantic salmon is the most commonly cultured finfish species in Newfoundland and Labrador (NL). In 2013, NL become the second largest producer in Canada with approximately 15% of total volume and value (Manning and Hubley, 2015). However, like in any intensive animal food producing sector, Atlantic salmon production in NL has had

to contend with disease outbreaks. Specifically, the Infectious Salmon Anaemia Virus (ISAV-HPR0 and ISAV NA-HPRΔ variants) which resulted in production losses for Atlantic salmon producers in 2014 (Fig. 1-3; Gagné, 2017); Atlantic salmon production volume decreased to 5,980 tonnes, accounting for 73.1 percent by volume of total production compared to 2013 (Government of Newfoundland and Labrador, 2014). Atlantic salmon production in NL, which is concentrated in the Bay d'Espoir and Fortune Bay regions, was reported as 14,167 tonnes in 2019 (Government of Newfoundland and Labrador, 2019). By 2024, Atlantic salmon production is expected to reach 50,000 tonnes (Government of Newfoundland and Labrador, 2019).

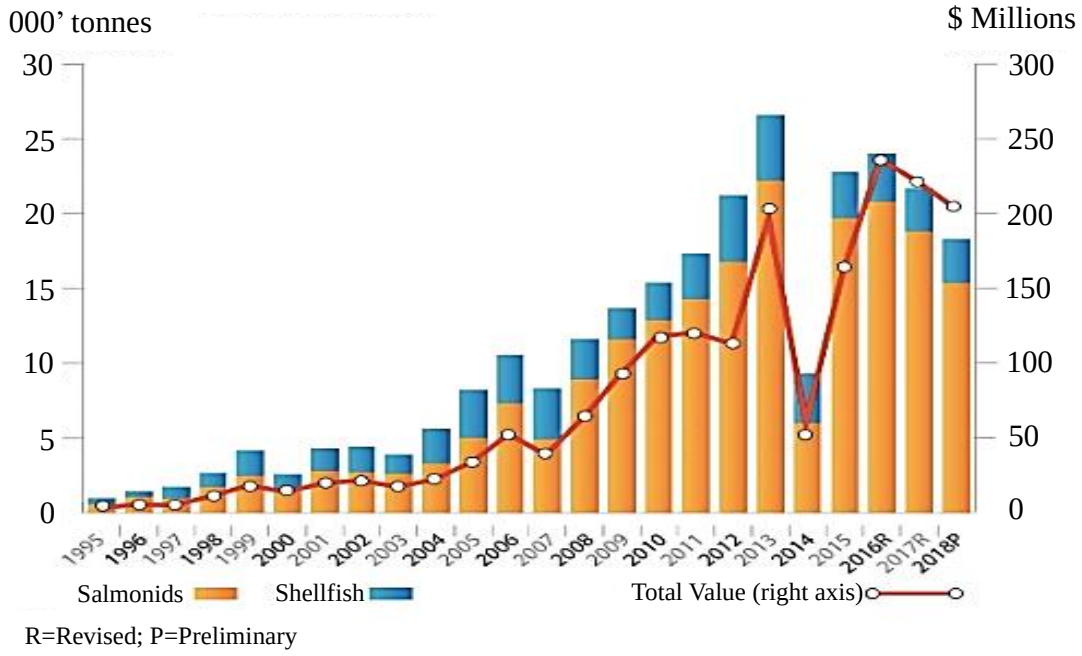

Figure 1-3. Aquaculture production in Newfoundland and Labrador for the period 1995-2018 (Government of Newfoundland and Labrador, 2018)

1.2. Sea lice infestation

Sea lice have negative consequences for the Atlantic salmon industry due to their impacts on salmon health, production yields, and the costs associated with monitoring and treatment. Estimated global economic losses to the global Atlantic salmon industry caused by sea lice are estimated at $460 million (CAD) annually (Costello, 2009; Erkinharju, 2020). Sea lice have a greater impact on salmon farming than other parasites (Costello et al., 2004). Sea lice are reported to negatively impact salmon by causing stress, changes in blood glucose or electrolytes, reduced haematocrits, reduced swimming performance, induced osmoregulatory dysfunction, physiological stress responses, anaemia, reduced feeding and growth, increased susceptibility to secondary microbial infections, reduced disease resistance and increased mortality (Wagner et al., 2003; Thorstad et al., 2008; Wagner et al., 2008; Finstad et al., 2011; Thorstad et al., 2015). Sea lice feed on the mucus, epidermal tissues, and blood of their hosts causing stress, wounds, and anemia in farmed and wild Atlantic salmon, which may lead to secondary infections, reduced immune response to opportunistic pathogens like ISAV (Barker et al., 2019) and osmoregulatory problems (Edvardsen et al., 2014; Thorstad et al., 2015; Helgesen et al., 2019; Umasuthan et al., 2020). The impact of sea lice on salmon depends on sea lice species, the number and stage of their development, and on the salmon species; for instance, pink salmon (*Oncorhynchus gorbuscha*) is more susceptible to sea lice than Chinook salmon and Chum salmon (*Oncorhynchus keta*) (Helle and Holm, 2017).

The salmon louse (*Lepeophtheirus salmonis*), an ectoparasite of the family Caligidae, is principally a parasite of salmonids. Two species of sea lice which present a major concern for Atlantic salmon farming are *Lepeophtheirus salmonis*, found in the

Northern Hemisphere, and *Caligus rogercresseyi* in the Southern Hemisphere (Johnson et al., 2004). *Lepeophtheirus salmonis* is larger, feeds more aggressively on its host, and is more pathogenic compared to *Caligus rogercresseyi* and other *Caligus* species. *C. rogercresseyi* is a species of sea lice found on more than 80 species of marine fish. It causes losses to the salmon industry in Chile, estimated up to 178 million US Dollars annually (FAO, 2008).

Sea lice outbreaks have been reported on salmonids every place they are farmed in the sea - Chile, Norway, Faroes, Iceland, Canada (BC & 4 Atlantic Provinces), Ireland, and Scotland (Leslie et al., 2004; Saksida et al., 2015; Thorstad et al., 2015) leading to control efforts through increased frequency of chemotherapy applications, vaccines, feed additives, selective breeding for Atlantic salmon with decreased susceptibility to sea lice, and more frequent use of cleaner fish (Raynard et al., 2002; Leslie, 2004; Jansen et al., 2012; Gharbi et al., 2015; Saksida, 2015; Thorstad et al. 2015; Núñez-Acuña et al., 2016). The use of chemotherapeutants like hydrogen peroxide, emamectin benzoate, organophosphates, pyrethroids, benzoyl phenylurea, and lufenuron to control lice can result in the development of resistance within sea lice (Aaen, 2015; Poley, 2018) and negative impacts on aquatic organisms and their environment (Burridge, 2013). Addressing lice infestations is one of the biggest concerns for the Atlantic Canadian aquaculture industry, and indeed all salmon farming jurisdictions (Marbase, 2020).

1.3.Cleaner fish

New methods for dealing with sea lice include warm-water treatments, freshwater bath treatment, hyposaline treatment, vaccines, cleaner fish, traps (either physical or biological), physical exclusion devices (nets, electrical fields), novel drugs for the treatment or removal of sea lice from salmon, immunological interference (immunostimulants), mechanical delousing systems, selective breeding for louse-resistant salmon and regulatory approaches (zones with synchronized production and fallowing) (Stone, 2002; Torrissen, 2013; Groner, 2019; Sievers, 2019; Hannisdal, 2020). Due to the fact that the long-term use of chemicals can lead to resistance development within the sea louse, and negative impacts on the culture environment (Aaen et al., 2015), Integrated Pest Management (IPM), which entails an effective approach to parasite management while minimizing risks to people and the environment, has been employed globally by the Atlantic salmon farming industry to manage sea lice (Brooks, 2009).

The use of cleaner fish species has re-emerged as a promising strategy to control sea lice (Imsland et al., 2014; Leclercq et al., 2014). Cleaner fish is considered a biological control strategy that has been documented as an alternative method for decreasing lice levels and reducing chemotherapeutic use in Atlantic salmon aquaculture (Treasurer, 2002; Powell et al., 2018). In the west North Atlantic, the two main species commonly employed are cunner (*Tautogolabrus adspersus*) and lumpfish (*Cyclopterus lumpus*) (Pampoulie, 2014; Umasuthan, 2021). Cunner and lumpfish are omnivores that have proven effective for the removal of sea lice from Atlantic salmon (Charmley, 2019). Although cunner and lumpfish are both used to control sea lice, they are quite different in terms of biology, ecology, and life history (Charmley, 2019). In contrast to other cleaner fish species,

lumpfish actively remove sea lice from farmed salmon in cold environments and they have been domesticated and industrialized in the North Atlantic region (Marcos-López et al., 2013; Imsland et al., 2014; Whittaker et al., 2018; Toffan et al., 2019).

Lumpfish (*Cyclopterus lumpus*) is a semi-pelagic fish with diverse habitats, while juveniles are thought to be mainly pelagic. The migration pathway of lumpfish is along coastal areas and they display a mix of pelagic/demersal behavior (Kennedy et al., 2018). At all stages, Lumpfish are often observed preventing drift in water currents by adhering to other objects. Lumpfish larvae hatch at approximately 5.6 mm standard length and develop rapidly to increase in length and weight to 1.3 mm and 7.1 mg in 33 days, respectively (Benfey and Medvan, 1986). Cultured lumpfish are commonly transferred to Atlantic salmon sea cages, where they reach a size of approximately 50–180 g (Imsland et al., 2014. Wild lumpfish (or lumpsucker) are widely distributed across a large area on both sides of the North Atlantic Ocean, from Nunavut, Hudson Bay, and Labrador, to New Jersey and Bermuda in the western Atlantic, to the Barents Sea, Iceland and Greenland and the Iberian Peninsula on the eastern side (Vasconcelos et al., 2004; Bañón et al., 2008; Pampoulie et al., 2014). They occur in high densities in the Bay of Fundy, New Brunswick and on the St. Pierre Bank off the south coast of Newfoundland (COSEWIC, 2017). Utilization and demand for lumpfish in salmon farms in Ireland, the UK, Norway, Faroes Islands, Iceland, and Canada have increased in recent years (Powell et al., 2018). In 2018, in Norway, approximately 40 million juvenile lumpfish were used (Imsland et al., 2018), in the UK, approximately 6 million lumpfish were used, in Iceland, approximately 3.5 million were used (Foss et al., 2020), and approximately 300 thousand lumpfish were used in Ireland (Bolton-Warberg, 2018). In Canada, the utilization of lumpfish is a more recent practice,

with approximately 1 million lumpfish being deployed to Atlantic salmon sea cage sites in Atlantic Canada in 2019 (Foss et al., 2020). Thus, the production of this species is very important for aquaculture in Canada (Torrissen et al., 2013).

1.4.Bacterial diseases of cleaner fish

One issue related to the cohabitation of cleaner fish with Atlantic salmon is the risk of disease transmission between the two species. Similar to other finfish species, lumpfish are susceptible to different types of bacterial pathogens such as *Vibrio spp*., atypical *Aeromonas salmonicida*, *Pasteurella spp*., *Tenacibaculum spp.*, *Pseudomonas anguilliseptica*, and *Moritella viscosa* (Gulla, 2015). Lumpfish are prone to bacterial diseases including *Aeromonas salmonicida* (causative agent of furunculosis in Atlantic salmon) and *Vibrio anguillarum* (causative agent of vibriosis) (Rimstad, 2017).

Vibriosis is an acute bacterial septicemia that negatively impacts fish welfare and results in economic losses for the aquaculture industry (Frans et al., 2011; Sudheesh et al., 2012). Vibrios are a diverse group of bacteria which include *V. anguillarum*, *V. ordalii*, *V. splendidus*, *V. tapetis*, *V. wodanis*, and *V. logeli*, *V. harveyii*, and *V. salmonicida* (Sudheesh et al., 2012; Nielsen et al., 2014). *V. anguillarum* is Gram-negative with a curved rodshaped, has a polar flagellum, and is a non-spore-forming, halophilic and facultative anaerobic bacterium (Austin and Austin, 2007; Frans et al., 2011; Holm et al., 2015). A total of 23 O-serotypes (O1–O23) displaying different pathogenicity have been identified. However, only serotypes O1, O2, and O3 are associated with vibriosis in fish (Pedersen et al., 1999). The symptoms of disease include dark skin lesions, ulceration, exophthalmia,

accumulation of fluid in the intestine, and swelling of the kidney and spleen. The virulence-related factors of *V. anguillarum* have been identified including chemotaxis and motility (Larsen et al., 1994), adhesions (Wang and Leung, 2000), invasion (Hickey, 2017), iron-sequestering systems (Crosa, 1980), secretion of extracellular enzymes, hemolytic and proteolytic extracellular products (Singer et al., 1991), lipopolysaccharide (Norqvist and Wolf-Watz, 1993), and serum resistance (Trust et al., 1981). Lumpfish (*Cyclopterus lumpus*), Pacific and Atlantic salmon (*Oncorhynchus spp.* and *Salmo salar*), Japanese flounder (*Paralichthys olivaceus*), rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), turbot (*Scophthalmus maximus*), and Atlantic cod (*Gadus morhua*) are susceptible to *V. anguillarum* infections (Ringø et al., 2007; Naka et al., 2011; Marcos-López et al., 2013; Rajan et al., 2013; Holm et al., 2015; Xing et al., 2019; Vasquez et al., 2020a).

1.5. Vaccination in Lumpfish (*C. lumpus*)

V. anguillarum and *A. salmonicida* cause significant mortality and economic effects (e.g., production losses and treatment costs) in both lumpfish and Atlantic salmon (Rønneseth et al., 2017; Brooker et al., 2018; Soto-Dávila et al., 2020). Wild-caught cleaner fish are more likely to be carriers of *V. anguillarum* and *A. salmonicida* that can pose the risk of possible disease transmission to salmon when held in cohabitation (Treasurer, 2002; Brooker et al., 2018). Vaccination against vibriosis and furunculosis is an important factor for the control of diseases and reducing the use of chemotherapeutics

in the Atlantic salmon aquaculture industry (Gjerde et al., 2009; Bruno et al., 2013; Ma et al., 2019).

Vaccines can be delivered to the fish via intraperitoneal injection (i.p.), immersion (by dipping or bath), and oral administration (Assefa and Abunna, 2018). Intraperitoneal vaccination is a widely employed immunization strategy affording a high level and duration of protection, however, this method is time-consuming and stresses the fish being vaccinated (Gould, 1978; Piganelli, 1994). Additionally, the requirement for large-scale vaccination in aquaculture and the size of fish at vaccination is also restrictive (Assefa and Abunna, 2018). Immersion vaccination has lower potency, shorter duration of immunity, but it is easier to apply in small fish, and less stressful compared to injection, while also being convenient for mass vaccination (Bøgwald and Dalmo, 2019). This method might allow antigen uptake across mucosal surfaces inducing both local and systemic immune responses (Huising, 2003; Sudheesh and Cain, 2017). Oral administration is perhaps the most desirable method of vaccine delivery (Gunnels et al., 1976). This method of vaccine administration is less stressful to the fish, and it provides an economic method for mass vaccination (Mutoloki et al., 2015). However, the level of protection afforded by oral vaccination has been inferior to other vaccination methods as antigens are often destroyed in the digestive system before they reach the sites where immune induction occurs (Embregts and Forlenza, 2016). Therefore, various encapsulation methods have been developed to protect antigens against gastric degradation (Quentel and Vigneulle, 1997; Mutoloki et al., 2015). Besides the route of vaccine administration, additional factors affect the immune response in fish, such as the nature of the antigen, the use of adjuvants, vaccine

dose, as well as fish age, size, and health status (Huising et al., 2003; Gudmundsdóttir and Björnsdóttir, 2007; Embregts and Forlenza, 2016).

Formalin-killed *V. anguillarum* serotypes O1 and O2 are often used to formulate vaccines for different fish species, including Atlantic salmon, Atlantic cod (*Gadus morhua*) (Mikkelsen et al., 2011), gilthead sea bream (*Sparus aurata*), and lumpfish (Vargas et al., 2018; Chakraborty et al., 2019). The efficacy of vaccines in lumpfish against *A. salmonicida* and *V. anguillarum* has been evaluated with variable results (Hansen, 2005; Bruno et al., 2013; Rønneseth et al., 2017). Three commercial vaccines have been approved against furunculosis and vibriosis in salmonids in Canada: Forte Micro® (*A. salmonicida – V. anguillarum – ordalii – salmonicida* bacterin); Forte VII® (Infectious Salmon Anaemia killed virus vaccine, *A. salmonicida, V. anguillarum – ordalii – salmonicida* bacterin); and Alpha JectMicro 4® (*A. salmonicida – V. anguillarum – Vibrio salmonicida* bacterin) (Boily et al., 2019). Vaccines against these diseases have been developed for Atlantic salmon, but optimal efficacy in lumpfish needs to be tested and improved. In addition, the knowledge of the immune system of the lumpfish, and its susceptibility and ability to resist infections, is limited, especially the larval stage, which is particularly vulnerable to infectious diseases due to the immature development of their immune system (Cui et al., 2018).

In recent years, lumpfish utilization in the Atlantic salmon aquaculture industry in Newfoundland and Labrador has increased significantly (Boyce et al., 2018; Marbase, 2020). However, outbreaks related to *V. anguillarum* have been reported (Vasquez, 2020a). Utilization of vaccines against local isolates, including the route of administration, in lumpfish needs to be studied and subsequently optimized.

Here, I propose to study the oral immunization route in larval and juvenile cultured lumpfish against *V. anguillarum* and provide new insights into vaccine utilization and efficacy in lumpfish. This research will contribute to the effective development of vaccines for use in cleaner fish and the optimization of their utility as a means of sea lice control. In addition, this research will contribute new insights regarding lumpfish immunity.

1.6. General research objectives

The general objective of this research is to evaluate the efficacy of oral immunization against *Vibrio anguillarum* in juvenile cultured lumpfish.

1.7. Specific research objectives

The specific objectives of this study are to: i) develop a bio-encapsulation method for *V. anguillarum* bacterin in *Artemia salina* nauplii*;* ii) evaluate the immune protection of orally immunized lumpfish larvae against *Vibrio anguillarum*; and iii) evaluate the gene expression profile of orally immunized whole body lumpfish larvae.

2. MATERIALS AND METHODS

2.1. *Vibrio anguillarum* J360 culture conditions

V. anguillarum J360 serotype O2 (NCBI IDs: Chromosome 1 CP034672; Chromosome 2 CP034673; and plasmid CP034674), a local lumpfish isolate, was used in this study (Vasquez, 2020). *V. anguillarum* J360 was grown in 3 mL of tryptic soy broth (TSB, Difco) for 24 h at 28 ^{o}C with aeration (180 rpm, in an orbital shaker). Bacterial growth was monitored using spectrophotometry (Genesys 10 UV spectrophotometer, Thermo Fisher Scientific Inc., Waltham, MA, USA) and by plating to determine the CFU mL^{-1}. When the optical density (OD600 nm) reached ~0.7 (1×10^8 CFU mL^{-1}), the cells were harvested by centrifugation at 4200× g for 10 min at room temperature. The cell suspension was washed twice with phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.2) (Sambrook, 2001) at 4200 x *g* and resuspended in 300 µL of PBS 1X (~1×10^{10} CFU mL^{-1}). The final inoculum was serially diluted (1:10), and the concentration was determined by plate counting (Leboffe, 2019) in TSB supplemented with 1.5% bacto agar (TSA).

2.2. Bacterin preparation

The bacterin preparation was conducted according to established protocols and quantified using flow cytometry enumeration (Eslamloo, 2020) with modifications. First, *V. anguillarum* J360 was grown in 500 mL of TSB supplemented with a final concentration of 150 µM 2,2-dipyridyl (Sigma-Aldrich, St. Louis, MO, USA) at 28 ^{o}C with aeration (180 rpm, in an orbital shaker) to induce synthesis of the iron-regulated outer membrane proteins (IROMPs) (Santander, 2012). Bacterial growth was monitored spectrophotometrically until

it reached ~1 × 10^8 CFU^{-1}. *V. anguillarum* cells were harvested using centrifugation (4200 x *g* at 4 ºC for 10 min) and washed twice with PBS. Cells were fixed with buffered formalin 6% (Sigma) at room temperature for 3 d with gentle agitation in a rocker shaker. Cell viability was determined each day by plating onto TSA. Formalin was removed using centrifugation, and cells were then dialyzed (Spectrum™ Spectra/Por™ dialysis membrane 12–14,000 Dalton molecular weight cut-off, Thermo) in 2 L of PBS at 6 ºC for 3 d with agitation. *V. anguillarum* bacterins were quantified using the BD FACS Aria II flow cytometer (BD Biosciences, San Jose, CA, USA) and BD FACS Diva v7.0 software as described previously (Vasquez, 2020). Bacterin cells (1 × 10^{10} CFU mL^{-1}) were stored at 4 ºC until use.

2.3. *V. anguillarum* bacterin fluorescent labelling

V. anguillarum bacterin was labeled with 5-([4,6-dichlorotriazinyl] amino) fluorescein hydrochloride (DTAF; Sigma) according to previously described protocols (Valderrama, 2019) with minor modifications. First, *V. anguillarum* bacterin (~1 × 10^{10} CFU mL^{-1}) was centrifuged at 4200 x *g* for 10 min and then resuspended in 950 µL of bicarbonate buffer (0.1 M, pH 9). Following that, the cells were mixed with 50 µL of DTAF solution (100 µg in dimethyl sulfoxide (DMSO); Sigma) and incubated overnight at 4 ºC in dark conditions. After incubation, the bacterin cells were centrifuged (4200 x *g* for 10 min) and washed three times with bicarbonate buffer and were finally resuspended in PBS and kept at 4 ºC until use.

2.4. Optimization of *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii

We describe the optimization of *V. anguillarum* bacterin bio-encapsulation in *A. salina*. To optimize bacterin bio-encapsulation in *A. salina*, we used the method described by Campbell et al. (1993) with modifications. Additionally, we developed a semi-quantitative method to estimate the levels of bacterin bio-encapsulation in *A. salina*. First, *A. salina* nauplii were hatched from cysts according to the supplier's instructions (INVE, Salt Lake City, UT, USA) (Fig. 2-1). After the *A. salina* nauplii hatched (~20 h at 20 ^{o}C), nauplii were washed with seawater for 30 min (Fig. 2-1). *A. salina* cultures were nutritionally supplemented with Ori-One (Skretting, Fontaine les Vervins, France) and Ori-Green (Skretting, Fontaine les Vervins, France) commercial dry microalgae extract at a ratio of 1:1 (Ori-One:Ori-green) for 3 h at 20 ^{o}C. To determine the optimal time for bacterin bio-encapsulation in *A. salina*, supplemented *A. salina* nauplii were inoculated into 6 well plates with a 3 mL total volume per well at a density of 1000 nauplii mL^{-1}. Additionally, a separate plate was inoculated with non-supplemented *A. salina* nauplii to determine the possible effect of nutrient supplementation on bio-encapsulation. Both groups were inoculated with DTAF-labeled *V. anguillarum* bacterin (5×10^{7} cells mL^{-1}). The control group was mock inoculated with seawater and used to evaluate autofluorescence. The nauplii were incubated at 20 ^{o}C for 48 h to determine the optimal time for bacterin bio-encapsulation (Fig. 2-1A). *A. salina* samples (1 mL) were collected at 1, 3, 5, 24, 36, and 48 h, and fixed in 10% buffered formalin. The presence of *V. anguillarum* inactivated bacterin in the larvae gut was examined and counted using confocal microscopy (Nikon Eclipse Ti, Melville, NY, USA) to determine the number of A. salina containing 0%, 25%,

50%, 75%, 100% bacterin in their gut (i.e.,where 100% was maximum gut capacity reached) (Fig. 2-1A).

After determining the optimal time for bacterin bio-encapsulation in *A. salina* nauplii, we evaluated the bio-encapsulation stability at 6 ºC for 6 d (Fig. 2-1B). *A. salina* nauplii were supplemented with Ori-One, Ori-Green, and DTAF-labeled *V. anguillarum* bacterin at 20 ºC for 3 h and then placed at 6 ºC for 6 d. The *A. salina* control group was mock inoculated with seawater. *A. salina* samples were collected each day and fixed with buffered 10% formalin. The levels of bio-encapsulation in *A. salina* were determined using confocal microscopy (40x) based on the fluorescence of bacterin present in the larvae gut (Fig. 2-1B).

A. salina nauplii with bio-encapsulated DTAF-labeled *V. anguillarum* inactivated bacterin were fed to lumpfish larvae (Fig. 2-2) to determine the presence of *V. anguillarum* bacterin in the larvae gut compared to the non-orally immunized fish (Fig. 2-3). Fifty lumpfish larvae were orally immunized and maintained at 6 ºC for 24 h. The larvae gut was observed at 0, 0.5, 1, 2, 4, 6, and 24 h post-oral immunization using epi-fluorescence microscopy (Optika, Italy) (Fig. 2-3).

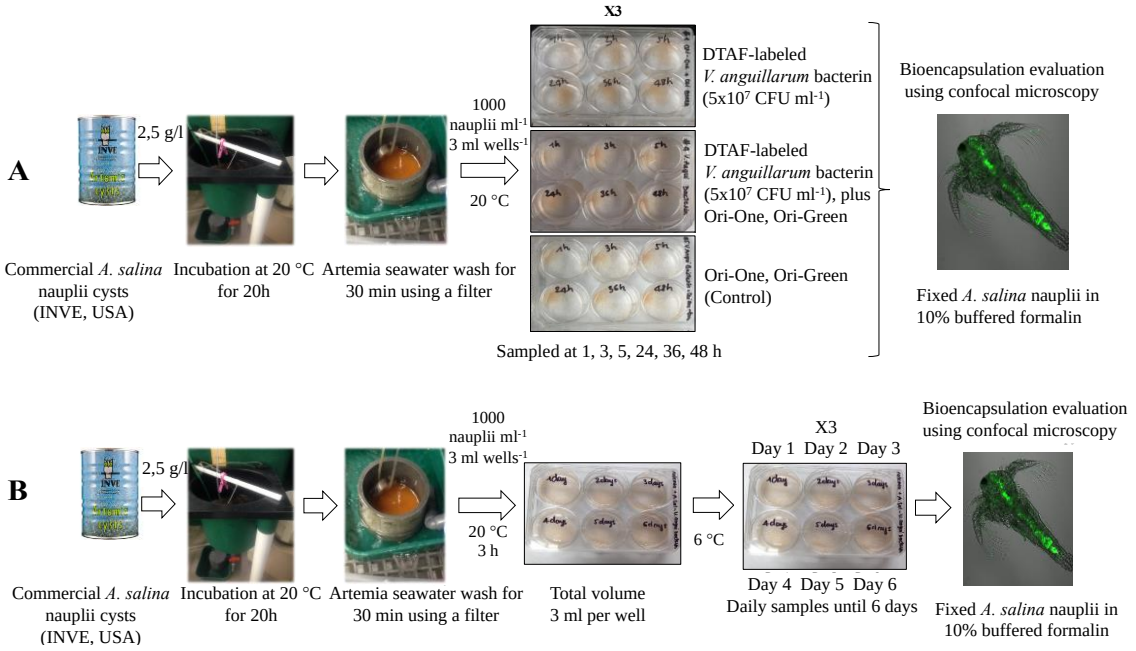

Figure 2-1. Experimental design for optimization of *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii. (A) Optimization of bio-encapsulation conditions and time. The effects of supplementation with Ori-One and Ori-Green on *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii were assessed. In addition, to determine the optimal incubation time for bio-encapsulation to occur, incubations were performed at 20 ^{o}C for either 1, 3, 5, 24, 36 or 48 h post-inoculation. The presence of the *V. anguillarum* bacterin in the *A. salina* nauplii intestine was then assessed using confocal microscopy. (B) The stability of the *V. anguillarum* bacterin in the intestine of *A. salina* nauplii post bio-encapsulation. Once the bio-encapsulation process was completed under the optimal conditions determined in (A), *A. salina* nauplii containing the *V. anguillarum* bacterin were stored at 6 ºC. The presence of the *V. anguillarum* bacterin in the *A. salina* nauplii intestine was then assessed daily for 6 days using confocal microscopy.

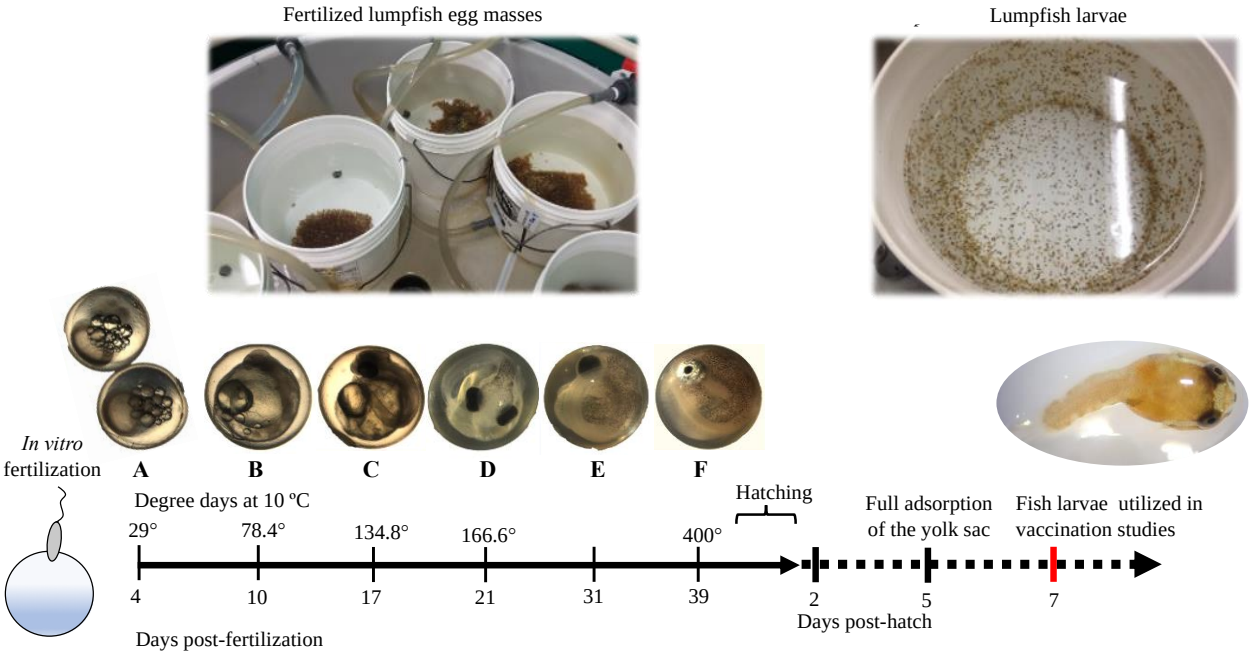

Figure 2-2. Lumpfish culture conditions and embryonic development. Fertilized lumpfish egg masses were maintained in 5 l buckets (see section 2.7 for details) during embryonic development: (A) Segmentation and compression of yolk lipids; (B) Embryo with oocysts and more developed eyes; (C) Eye pigmentation and otoliths; (D) Skin pigmentation; (E) The embryo's body; (F) Large embryo ready to hatch. At 7 dph, the larvae were subjected to vaccination studies.

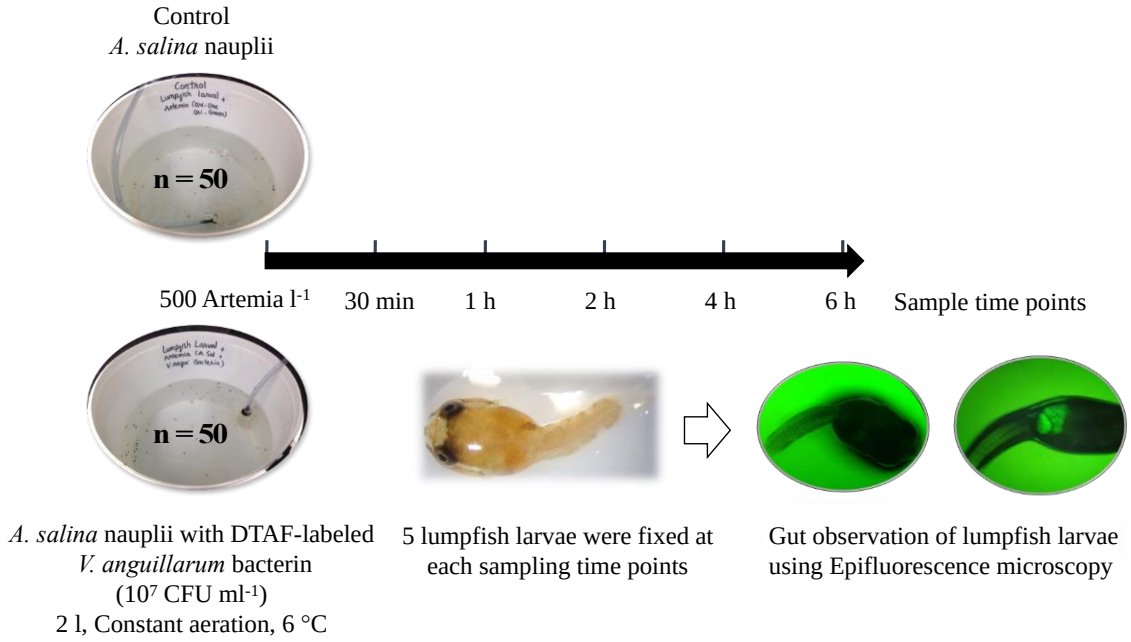

Figure 2-3. Oral immunization of lumpfish *(Cyclopterus lumpus)* larvae with the DTAF-labeled *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii. Lumpfish larvae (7 dph) were either fed *A. salina* nauplii with the bio-encapsulated DTAF-labeled *V. anguillarum* bacterin or control *A. salina* nauplii that had been inoculated with seawater, and maintained at 6 °C for 12 h. The presence of *V. anguillarum* bacterin in the gut of lumpfish larvae compared to non-orally immunized fish was then assessed at 0, 0.5, 1, 2, 4, and 6 h post-oral immunization by fluorescence microscopy.

2.5. *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii

Based on the vaccine bio-encapsulation optimization results, larger volumes of *A. salina* were prepared for lumpfish larvae immunization. *A. salina* nauplii were hatched and

washed with seawater for 30 min and placed in 20 L buckets containing 15 L of seawater. *A. salina* nauplii were maintained at a density of ~2.5 million *A. salina* per liter. *A. salina* cultures were enriched with nutritional supplements derived from microalgae, OriOne (Skretting, France) and Ori-Green (Skretting, France) according to the manufacturer's instructions. *A. salina* cultures were inoculated with *V. anguillarum* bacterin (10^7 cells mL^{-1}) and incubated at 20 ºC for 3 h with aeration. Controls were mock inoculated with PBS. After 3 h of enrichment and bacterin bio-encapsulation, the *A. salina* cultures were maintained at 6 ºC for 6 d under constant light. These cultures were used for the daily oral immunization of the lumpfish larvae (Fig. 2-4).

Figure 2-4. *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii for industrial application.

2.6. Aquafeed coating with *V. anguillarum* bacterin

Commercial dry feed was coated with dry *V. anguillarum* bacterin to orally boost immunized fish. Ficoll, a non-toxic polymer, was used as a cryoprotectant for bacterial lyophilization (Wessman, 2013). Ficoll also serves as an antigen and adjuvant carrier (Inman, 1975; Anderson, 1983; Amlot, 1986; Fissell, 2007; Wessman, 2013; Milley, 2016). To freezedry the bacterin, a formalin-killed *V. anguillarum* (2×10^9 CFU mL^{-1}) suspension was mixed with Ficoll solution (20% Ficoll400 (GE Healthcare, Sweden), 300 mM NaCl) at a 1:1 ratio to prevent cell lysis and then lyophilized. The cells were lyophilized (Edwards super module E2-M5, Edwards, UK) for 3 days. The bacterin powder was mixed with 3–4 mm commercial dry pellet (Skretting-Europa 15: crude protein (55%), crude fat (15%), crude fiber (1.5%), calcium (3%), phosphorus (2%), sodium (1%), vitamin A (5000 IU kg^{-1}), vitamin D (3000 IU kg^{-1}), and vitamin E (200 IU kg^{-1}) at the ratio of 0.9 g bacterin per 100 g aquafeed. After mixing the feed with the bacterin powder, a layer of cod liver oil was added (3 mL 100 g $feed^{-1}$), and the feed was then dried at room temperature to complete the coating process. The coated feed was stored at 4 ^{o}C until used.

2.7. Fish culture conditions

All animal protocols required for this research were approved by the Institutional Animal Care Committee and the Biosafety Committee at Memorial University of Newfoundland (MUN). Experiments were conducted under protocols #18-01-JS, #18-03-JS, and biohazard license L-01. Lumpfish egg masses were in vitro fertilized and maintained in 5 L buckets containing UV treated (300 mW cm^{-2}), filtered, flow-through

seawater (33 ppt) at 10–10.5 ºC, with 95–110% air saturation, and held under ambient photoperiod (spring– summer) at the Joe Brown Aquatic Research Building (JBARB), Department of Ocean Sciences, Memorial University of Newfoundland, Canada. After embryo development was complete, the larvae were hatched and maintained in the same seawater conditions and air saturation at 10 ºC until full egg yolk sac absorption and the establishment of independent feeding was achieved (Fig. 2-2).

2.8. Lumpfish immunization assays

Lumpfish larvae were stocked in 4 tanks at a density of 2000 larvae per tank (20 L) with UV treated (300 mW cm^{-2}), filtered, flow-through seawater at 8–10 ºC, and with 95–110% air saturation (Fig. 2-5). Two tanks containing lumpfish larvae (1-week post-hatch (wph)) were orally immunized with the bio-encapsulated *V. anguillarum* vaccine daily for 4 weeks. Two tanks fed containing only *A. salina* served as controls (Fig. 2-5). Lumpfish larvae were fed with *A. salina* nauplii or bio-encapsulated vaccine 3 times per day (350 mL of *A. salina* culture per 2000 larvae) (Fig. 2-4). Thereafter, the fish were fed *A. salina* for additional 10 d and then fed with dry pelleted diet daily (0.75–2% body weight). Whole larvae pool samples were collected at 0, 2, and 4 weeks post-immunization (wpi) (Fig. 2-5). At each time point, triplicate pools of 5–10 larvae were sampled from each tank and placed in a 1.5 mL RNase-free tube containing 300 µL of TRIzol Reagent (Invitrogen, Waltham, MA, USA), flash-frozen in liquid nitrogen, and stored at −80 ºC until processing. After 8 weeks, the juvenile lumpfish were transferred into eight 500 L tanks (Fig. 2-5). The lumpfish were fed an assorted size pelleted diet for the remainder of the experiment (15 months). Nine months post-hatch, two tanks with 100 fish each (~72.1 ± 30 g) were orally

boost immunized using commercial pellets coated with the *V. anguillarum* bacterin. Lumpfish were starved for 24 h pre-oral vaccination. Lumpfish were fed with *V. anguillarum* bacterin coated dry pellets three times (every 2 weeks for 3 days) at 0.75% body weight (Fig. 2-5). Two control tanks were mock-orally boosted with ficoll (vaccine vehicle) coated pellets (Fig. 2-5). Two independent groups orally boosted were additionally i.p. boosted at 40 wph (~145 ± 31.8 g) with *V. anguillarum* bacterin (6.3×10^8 cells dose^{-1}). Control groups were mock-orally and i.p. boosted with the respective vaccine vehicle (Fig. 2-5). Two months later, the animals (~132–244 g) were transferred to the AQ3 aquatic biocontainment facility at the Cold-Ocean Deep-Sea Research Facility (CDRF) for challenge assays.

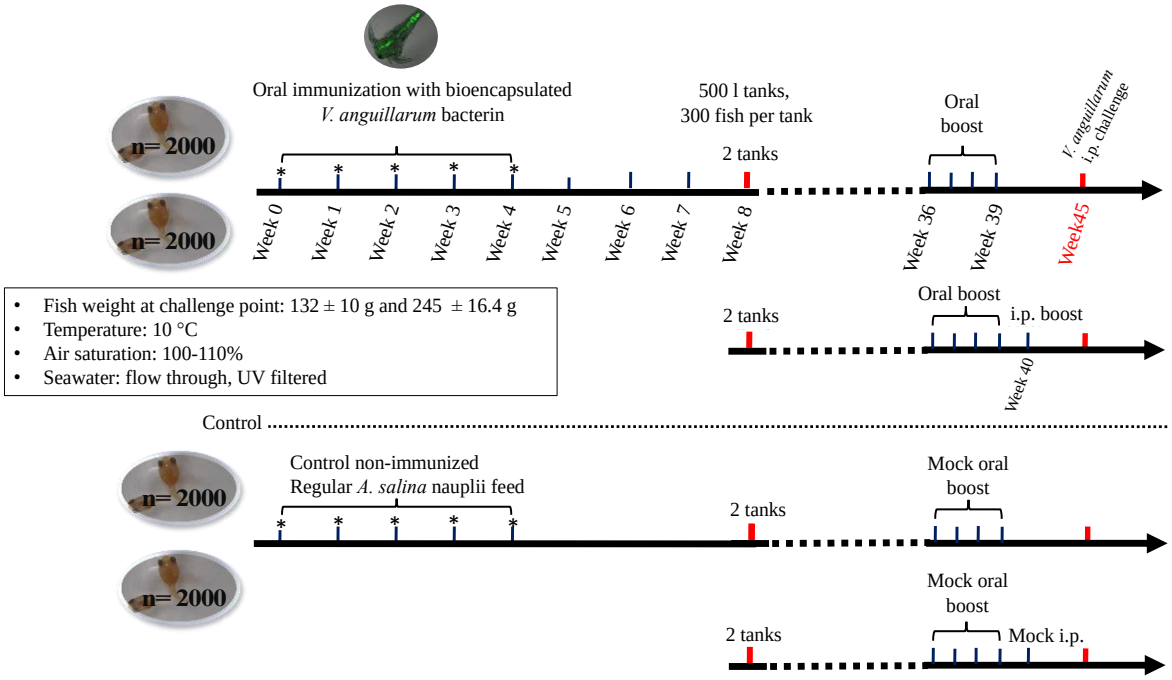

Figure 2-5. Immunization and challenge experimental design. Lumpfish larvae were immunized at 1 wph. At 8 wph, the fish were distributed in 500 L tanks. Lumpfish were boosted at 36 wph and challenged with *V. anguillarum* at 45 wph. *Larvae samples were collected at weeks 0, 2, 4 post-immunization for RNA extraction.

2.9. *V. anguillarum* J360 challenge assays in lumpfish

The challenge assays were conducted at the CDRF AQ3 biocontainment facility under the Institutional Animal Care Committee approved protocol (18-02-JS) and established protocols (Amend, 1981; Chakraborty, 2019). First, after transfer to the AQ3 biocontainment facility, lumpfish were acclimated for 1 week under optimal conditions prior to the commencement of the challenge. Lumpfish were challenged by an i.p. injection with 7 times the lethal dose 50 (7.8×10^5 CFU dose^{-1}) of *V. anguillarum* J360. Fish survival

was monitored for 30 days post-challenge. The relative percent of survival (RPS) of the control and vaccinated fish was calculated using the formula: RPS = [1 − (% mortality in vaccinated fish/% mortality in control fish)] × 100 (Amend, 1981).

2.10. Total RNA extraction

RNA was extracted from the larvae pools pre-immunized (n = 3 individual pools of 10 larvae each), 2 wpi (n = 3 pools of 10 larvae each), and 4 wpi (n = 3 pools of 5 larvae each). RNA was also extracted from the larvae post mock immunization (control) at 2 and 4 wpi. Lumpfish larvae pools, previously flash frozen in a 1.5 mL RNase-free tube containing 300 µL of TRIzol reagent (Invitrogen), were homogenized using a micro-tube homogenizer (ThermoFisher Scientific, Waltham, MA, USA). An additional 700 µL of TRIzol was added to the tube, and the extractions were then completed following the manufacturer's instructions. The TRIzol extracted samples were then purified using the RNeasy® Mini Kit (QIAGEN, Mississauga, ON, Canada) following the manufacturer's instructions. RNA samples were treated with 2 U mL^{-1} of TURBO DNase (TURBO DNAfree™ Kit, Invitrogen) following the manufacturer's instructions for the complete digestion of genomic DNA and the removal of the remaining DNase and divalent cations, such as magnesium and calcium. Purified RNA samples were quantified and evaluated for purity using a Genova Nano spectrophotometer (Jenway, Staffordshire, UK) and evaluated for integrity using 1% agarose gel electrophoresis (Sambrook, 2001). A PCR test was conducted using the 60S ribosomal protein L32 (rpl32) reference reference gene primers and the RNA as a template to rule out the presence of DNA.

1

Table 2-1. Sequences of primer pairs used in qPCR analyses of transcript expression levels in lumpfish (*Cyclopterus lumpus*) larvae.

Gene name (symbol)[a]	Trinity ID from NCBI SRA acc. no. SRP238224	Nucleotide sequence (5'-3')	Amplicon Size (bp)	Efficiency (%)
C-C motif chemokine-like 19 (*ccl19*)	DN10492_c0_g1_i4	F: GCTCAGGTACCAACGGACTG R: CGTGTCCTCCGATCTGTCTC	94	94.33
cyclooxygenase-2 (*cox2*)	DN750_c1_g1_i1	F: GAATTCCTCACCTGGGTCAA R: ATGGCATCTCTGAGGAAGGA	122	99.18
hepcidin anti-microbial peptide (*hamp*)	DN2993_c0_g1_i4	F: GCTCGCCTTTATTTGCATTC R: ATATGCCGCAACTGGAGTGT	100	93.36
interleukin 8_a (*il8a*)	DN21169_c0_g1_i2	F: AAGTCATAGCCGGACTGTCG R: CCCTGCTGATGGAGTTGTCT	109	100.39
interleukin 8_b (*il8b*)	DN4613_c0_g1_i4	F: GTCTGAGAAGCCTGGGAGTG R: TCAGAGTGGCAATGATCTCG	138	98.15
interleukin 10 (*il10*)	DN41536_c0_g1_i1	F: AACCAGTGCTGTCGTTTCGT R: TGTCCAAGTCATCGTTTGCT	106	95.24
ATP-dependent RNA helicase lgp2 (*lgp2*)	DN49186_c0_g1_i1	F: GCAACCTGGTGGTACGCTAT R: CTCGGCGACCACTGAATACT	104	81.54
interferon-induced GTP-binding protein_a (*mxa*)	DN526_c0_g1_i6	F: TGCACAGACTCAAGCAGAGC R: CCACACTTGAGCTCCTCTCC	144	85.43
interferon-induced GTP-binding protein_b (*mxb*)	DN526_c0_g1_i3	F: TTGCGGCTTGGAAAAATATC R: TCCACGGTACCTTCGTTCAT	95	92.78
interferon-induced GTP-binding protein_c (*mxc*)	DN237_c1_g1_i1	F: GGAAGTGGCAGACATTGTGA R: CTGCTGCAATCTCCTTCTCC	131	90.70
signal transducer and activator of transcription 1 (*stat1*)	DN3250_c2_g1_i2	F: CTCAAGATGCTGGACTGCAA R: ATGCTCTCGATCCACTTGCT	104	84.99
toll-like receptor 3 (*tlr3*)	DN30532_c0_g1_i1	F: AGAGGGCAGGGAATTTGAGT	101	90.29

		R: TGCACGAGTCATTCTCCAAG		
C-C motif chemokine-like 20 (*ccl20*)	DN9266_c0_g1_i3	F: ATGGGCTACACCATCCAGAC	102	80.07
		R: CCACTTGGATGAAGGGTCAG		
immunoglobulin heavy chain variable region a (*igha*)	DN1665_c0_g3_i2	F: AGGACTGGAGTGGATTGGAA	129	91.00
		R: TGCATGGTCTGTCCGTTTAG		
immunoglobulin heavy chain_b (*ighb*)	DN1665_c0_g4_i1	F: GAATGGAACAAGGGGACAAA	108	90.60
		R: CGGTCGTTGAGTCTCTCCTC		
interferon regulatory factor 7 (*irf7*)	DN6933_c0_g1_i2	F: GGCTCATAGAGCAGGTGGAG	115	81.89
		R: CTGTCTTCGTCGTTGCAGTC		
HLA class II histocompatibility antigen gamma chain (*cd74*)	DN13708_c0_g1_i6	F: ACGCCAAGACACCTCTGACT	108	96.45
		R: GGAAGGTCTCGTTGAACTGC		
serum amyloid A 5 (*saa5*)	DN41536_c0_g1_i1	F: AGAGTGGGTGCAGGAAAGAA	116	95.9
		R: GAAGTCCTGGTGGCCTGTAA		
T-cell surface glycoprotein CD4_a (*cd4a*)[c]	DN9678_c0_g2_i9	F: CGTTAAGGTGCTGCAGATCA	122	84.85
		R: GCGGAAACCATTTCAGTTGT		
T-cell surface glycoprotein CD4_b (*cd4b*)[c]	DN24146_c0_g1_i7	F: TGTGGGGTTAGCTCCTTCAC	138	94.24
		R: TGTTTGCGATCTCACCTTTG		
interleukin 1 beta (*il1b*)[c]	DN22448_c0_g2_i1	F: ATTGTGTTCGAGCTCGGTTC	98	97.37
		R: CGAACTATGGTCCGCTTCTC		
toll-like receptor 5_a (*tlr5a*)[c]	DN29432_c0_g1_i1	F: TGGACGAGTTTCAGCAGTTG	129	95.58
		R: AGACCCCTCACATGTCCAAG		
toll-like receptor 5_b (*tlr5b*)[c]	DN55824_c0_g1_i5	F: CCATCATGCACTTTGTACGG	127	88.57
		R: TGCTGTTGATCTCCCTGATG		
tumour necrosis factor alpha (*tnfa*)[c]	DN26791_c0_g1_i1	F: TTAGAAGGGAGCTGCGAAGA	119	90.06
		R: ATGACGATCCGGTTGTTCTC		
lymphocyte antigen 6 complex locus protein G6f (*ly6g6f*)[c]	DN12606_c0_g1_i8	F: TCCATGTGGACGTGACTGTT	100	88.17
		R: AACGGTGTCTGAGCCTGAGT		
T-cell surface glycoprotein CD8 alpha chain (*cd8a*)[c]	DN11791_c0_g1_i1	F: GCTTTGCTCTCTGGGCATAC	104	89.62
		R: TCCGGGTTCTTAAGTGGTTG		
immunoglobulin mu heavy chain_a (*ighma*)[c]	DN121_c0_g3_i3	F: CAGCTTCTGGATTAGACTTTGA	107	90.17

		R: GATGTTGTTACTGTTGTGTTGG		
immunoglobulin mu heavy chain_c (*ighmc*)[c]	DN121_c0_g3_i4	F: CAACATCCGGAATCACATTCAG R: GATTTTGAGGTCCCACTACCAT	112	87.68
interferon gamma (*infg*)[c]	DN81754_c0_g1_i1	F: CTCTGGCTGGTTGTCTGTCA R: TCGCTCTCTCGATGGAATCT	105	90.75
immunoglobulin delta heavy chain (*ighd*)[c]	DN1665_c0_g2_i7	F: GGAGACAGTGTTGTGCTGGA R: GGGCTTCAGGAAATTCAACA	121	88.41
toll-like receptor 7 (*tlr7*)[c]	DN760_c1_g2_i1	F: GGCAAACTGGAAGAATTGGA R: GAAGGGATTTGAGGGAGGAG	100	90.55
radical S-adenosyl methionine domain containing protein 2 / viperin (*rsad2*)[c]	DN16769_c0_g1_i1	F: AGGAGAGGGTGAAGGGAGAG R: ATCCAGAGGCAGGACAAATG	133	98.47
Normalizer[b]				
eukaryotic translation initiation factor 3 subunit D (*etif3d*)	DN7623_c0_g1_i5	F: AGCCAGATCAACCTGAGCAT R: AGGCTGTACACCCGAATCAC	134	86.49
60S ribosomal protein L32 (*rpl32*)	DN3569_c0_g1_i2	F: GTAAGCCCAGGGGTATCGAC R: GGGCAGCATGTACTTGGTCT	107	80.08
elongation factor 1 alpha_a (*ef1a_a*)	DN12280_c0_g1_i3	F: CAAGGGATGGAAGATTGAGC R: TGTTCCGATACCTCCGATTT	151	83.81
elongation factor 1 alpha_c (*ef1a_c*)	DN12280_c0_g1_i4	F: AAGCGCTTTGAGGAAATCACC R: GCTCGACCTTCCAACTCTTG	160	95.60
polyadenylate-binding protein 1_a (*pabpc1_a*)	DN6565_c0_g2_i3, DN6565_c0_g2_i4	F: CAAGAACTTTGGGGAGGACA R: TGACAAAGCCAAATCCCTTC	125	84.76
polyadenylate-binding protein 1_b (*pabpc1_b*)	DN6565_c0_g2_i5	F: GACTCAGGAGGCAGCTGAAC R: TCGCGCTCTTTACGAGATTT	102	88.11

*4-pt standard curve; [a]Expression levels of the transcripts of interest were normalized to expression levels of these two transcripts; [b]Candidate endogenous control transcripts; [c]Expression levels of these transcripts were low in lumpfish larvae; efficiencies are those reported for lumpfish head kidney (Gnanagobal et al., submitted).

2.11. cDNA synthesis and qPCR parameters

cDNA was synthesized in 20 µL reactions from 1 µg of RNA using SuperScript IV VILO Master Mix (Invitrogen) following the manufacturer's instructions. PCR amplifications were performed in 13 µL reactions using 1X Power SYBR Green PCR Master Mix (AppliedBiosystems, Waltham, MA, USA), 50 nM of both the forward and reverse primers, an indicated cDNA quantity. Amplifications were performed using the QuantStudio 6 Flex Real-Time PCR system (384-well format) (Applied Biosystems, Waltham, MA, USA). The real-time analysis program consisted of 1 cycle of 50 ^{o}C for 2 min, 1 cycle of 95 ^{o}C for 10 min, 40 cycles of 95 C for 15 s, and 60 ^{o}C for 1 min, with fluorescence detection at the end of each 60 ^{o}C step and followed by dissociation curve analysis.

2.12. qPCR primer quality assurance testing

All primer pairs for the transcripts of interest (TOIs) that related to innate and adaptive immune response and the endogenous control transcripts were designed and quality control (QC) tested using the larvae RNA samples generated herein. cDNAs were synthesized from the individual pooled larvae RNA samples, including pre-immunized control, mock-immunized control (2 and 4 wpi), and immunized larvae (2 and 4 wpi) to determine the efficiency of primers and Ct values. The control and immunized cDNA samples were independently pooled and used for primer quality evaluation. To calculate amplification efficiencies for each primer pair (Pfaffl, 2001), standard curves were generated for both cDNA pools (control and immunized) using a 5-point 1:3 dilution series

starting with cDNA representing 10 ng of input total RNA. The reported efficiencies represent an average of the two values (Table. 2-1). Each primer pair was also tested to ensure that a single product was amplified and that there was no primer dimer present in the no-template control. Finally, amplicons were electrophoretically separated on 2% agarose gels and compared using a 1 kb plus ladder (Invitrogen) to verify that the correct size fragment was amplified. Eighteen TOIs were well expressed in larvae, and as such, amplification efficiencies could be calculated using larvae cDNA template (see fluorescence threshold cycle (C_T) values for studies 1 to 3; Appendix I). However, fourteen of these transcripts were expressed at low levels in larvae (see C_T values for studies 4 to 6; Appendix I). For the latter TOIs, technical replicates and spacing were acceptable over the first three points of the cDNA dilution series. As the experimental input cDNA amount (8 ng) for these TOIs lies within the first 2 dilutions, these assays were deemed acceptable for analysis in larvae. However, for these fourteen transcripts, the amplification efficiencies reported in Table 2-1 and inputted into the QuantStudio Real-Time PCR Software (version 1.3) (Applied Biosystems) were those that had been previously generated for head kidney samples, due to the fact that the efficiency of some primers of the larvae samples could not be determined.

2.13. Endogenous control (normalizer) selection

Expression levels of the TOIs were normalized to transcript levels of two endogenous controls. To select these endogenous controls, 5 transcripts (*rpl32*, *elongation factor 1-alpha (ef1a), eukaryotic translation initiation factor 3 subunit D* (*etif3d*), *polyadenylate-binding protein 1a* (*pabpc1_a*) and *polyadenylate-binding protein 1b* (*pabpc1_b*)) were

analyzed. Briefly, the C_T values of all 27 samples in the study were measured (in duplicate) for each of these transcripts using cDNA representing 3.25 ng of input total RNA, and then analyzed using geNorm (Vandesompele et al., 2002). Based on this analysis, *rpl32* (geNorm M = 0.169) and *etif3d* (geNorm M = 0.177) were selected as the two endogenous controls.

2.14. Experimental qPCR analyses

To study the effects of oral immunization with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* on the immunome of lumpfish larvae, expression levels of 32 TOIs with immunerelevant functional annotations were assessed (Appendix I). Individual larvae pools (n = 27 pools) were subjected to qPCR analyses (Figure 1). In the qPCR analyses, cDNA representing 3.25 ng (study 1 to 3, Appendix I) and 8 ng (study 3 to 6, see Appendix I) of input RNA was used as a template in the PCR reactions. The input RNA concentration was increased to 8 ng due to lower expression transcript levels in the larvae. In each qPCR study, expression levels of a given transcript were measured across two plates. On each plate, the TOIs and endogenous controls were tested in triplicate, and a notemplate control was included for every sample. A plate linker sample was also included to ensure that there was no plate-to-plate variability. The relative quantity (RQ) of each transcript was determined using the QuantStudio Real-Time PCR Software (version 1.3), with normalization to both the *rpl32* and *etif3d* transcript levels and with the amplification and efficiencies incorporated (Appendix III). For each TOIs, the sample with the lowest normalized expression (mRNA) level was set as the calibrator sample (i.e., assigned an RQ value = 1) (Appendix I). Additionally, the transcript expression levels were determined

using the comparative $2^{-\Delta\Delta Ct}$ method (Livak,2001) with two reference genes (Soto-Davila, 2019) (Appendix II).

2.15. Statistical analysis

All data are expressed as the mean ± standard error (SE). Assumptions of normality and homogeneity were tested for variances. A one-way ANOVA followed by Tukey's multiple comparison post hoc test was used to determine significant differences between the survival of the control and infected groups. The Kaplan–Meier estimator was applied for the estimation of the survival fractions after the *V. anguillarum* challenges, and the log-rank test was used to identify differences between treatment groups ($p < 0.0001$). A twoway ANOVA was used to analyze the gene expression data followed by Sidak's multiple comparisons post hoc test to identify significant differences between each treatment in the control and immunized groups at each time point (2 weeks and 4 weeks). All statistical tests were performed using Graphpad Prism version 8.0 (Graphpad Software, USA, www.GraphPad.com (accessed on 15 June 2021)), and *p*-values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Bio-encapsulation of *V. anguillarum* bacterin in *A. salina* nauplii

A semi-quantitative method (a method that approximates the level of bacterin in the gut of *Artemia* based on the image produced by the fluorescence microscope) was established to estimate the levels of *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii (Fig. 3-1A). Autofluorescence was ruled out using *A. salina* nauplii inoculated with nutritional supplements (Ori-One and Ori-Green) (Fig. 3-1B). Using this method, we determined that approximately 100% of the A. salina nauplii reached maximum capacity (e.g., gut was completely full of bacterin) for *V. anguillarum* bacterin bio-encapsulation 3 h post-inoculation at 20 ^{o}C in both the absence (Fig. 3-1C) or presence (Fig. 3-1D) of nutritional supplements. In the absence of supplements, a significant decrease in the percentage of the *A. salina* nauplii with 100% bio-encapsulation levels was observed 24 h post-inoculation, which gradually declined thereafter (Fig. 3-1C). In the presence of nutritional supplements, a significant decrease occurred 36 h post-inoculation (Fig. 3-1D). We determined that the optimal *V. anguillarum* bacterin bio-encapsulation in *A. salina* method is the presence of nutritional supplementation with a bio-encapsulation time of 3 h at 20 ^{o}C. As we wanted to produce a bio-encapsulated vaccine batch that could be used for several days, we evaluated the stability of the *V. anguillarum* bacterin in the intestine of *A. salina* nauplii post bio-encapsulation at 6 ^{o}C (Fig. 3-1E). This temperature was chosen as it is similar to the water temperature at which lumpfish are cultured. We determined that the bacterin concentration in *A. salina* nauplii remained stable for at least 6 d post-inoculation (Fig. 3-1E). DTAF-labeled *V. anguillarum* bacterin bio-encapsulated in *A.*

salina nauplii were then fed to lumpfish larvae and fluorescence microscopy was used to determine if the *V. anguillarum* bacterin reached the larvae gut (Fig. 3-2). Fluorescence microscopy demonstrated the presence of the *V. anguillarum* bacterin in the gut of the lumpfish larvae after 6 and 24 h, whereas fluorescence was not detected in the gut of lumpfish larvae who had been fed *A. salina* nauplii only (Fig. 3-2).

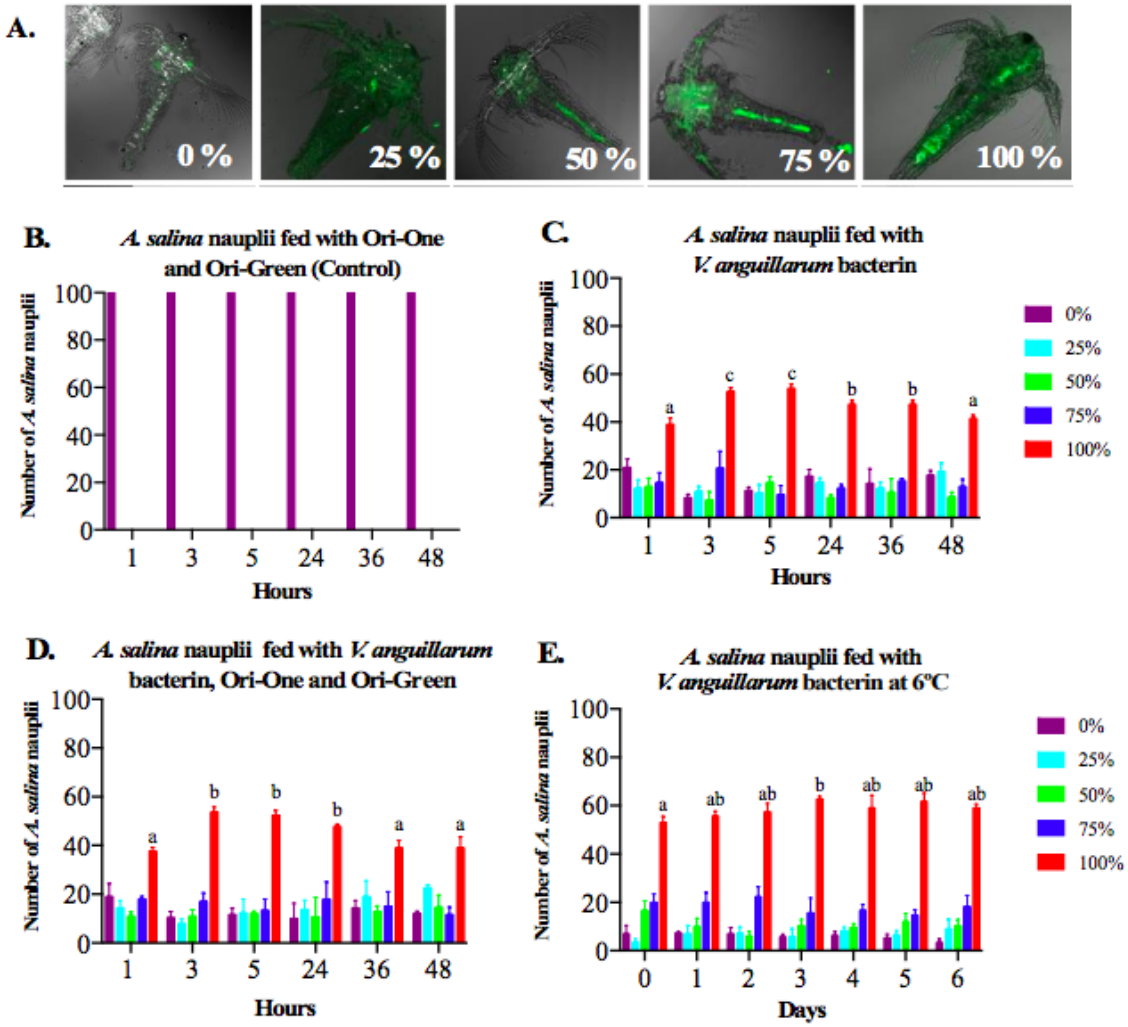

Figure 3-1. Optimization of *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii. (A). Relative percentage of DTAF-labeled *V. anguillarum* bacterin in *A. salina* nauplii intestine; (B) *A. salina* nauplii supplemented with dry microalgae (Ori-One and Ori-Green) as autofluorescence control. *A. salina* fed with commercial dry microalgae (Ori-One and Ori-Green) at 20 °C; (C) *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii at 20 °C; (D) *V. anguillarum* bacterin and commercial dry microalgae bio-encapsulation in *A. salina* nauplii at 20 °C; (E) bio-encapsulation stability at 6 °C after 3 h post enrichment

with *V. anguillarum* bacterin at 20 °C. In all cases, different color bars represent the % bio-encapsulation levels of the *V. anguillarum* bacterin in *A. salina* nauplii. Each value is the mean ± SEM for 3 groups of 100 A. salina nauplii per group. Different letters (a, b, c) indicate the differences in the numbers of A. salina nauplii enriched with 100% V. anguillarum bacterin at different time points. Means with different letters differ significantly ($p < 0.05$). Bars represent mean ± SEM.

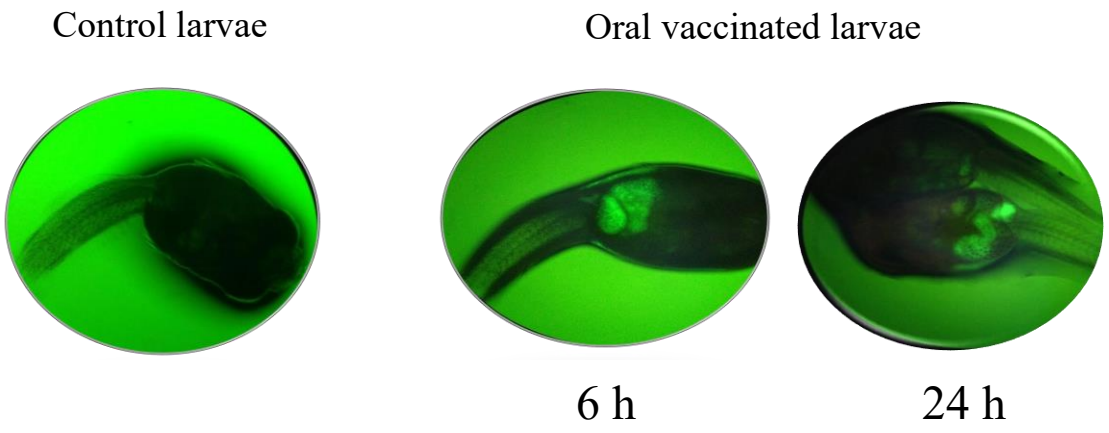

Figure 3-2. Selected images depicting the DTAF-labeled *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii in the lumpfish gut after 6 and 24 h, visualized using epifluorescence microscopy. Its presence is indicated by green fluorescence

3.2. Transcript expression profile of the immunome of orally immunized lumpfish larvae

Expression levels of the transcripts related to the innate and adaptive immune response were measured in pre-immunized larvae and at 2 and 4 wpi with the *V.*

anguillarum bacterin bio-encapsulated in *A. salina* nauplii. The transcript expression levels were analyzed using both the $2^{-\Delta\Delta Ct}$ (Figs. 3-3 to 3-5) and the RQ methods (Figs. 3-6 to 3-8). Both methods showed similar results, with the exception of the statistical significance demonstrated for *toll-like receptor 7* (*tlr7*) and *immunoglobulin heavy chain b* (*ighb*) (Figs. 3-3I, 3-4D; Figs. 3-6I, 3-7D). *Tlr7* showed statistical significance as determined by the RQ method, while no significant differences of this gene were found by the $2^{-\Delta\Delta Ct}$ method. In the case of *ighb*, the significance level occurred at 2 wpi as determined by the $2^{-\Delta\Delta Ct}$ method, whereas it was significant at 4 wpi according to the RQ method (Figs. 3-3I, 3-4D; Figs. 3-6I, 3-7D). Transcript expression levels were compared statistically in orally immunized compared to control larvae at 2 and 4 wpi only. Comparisons over time could not be assessed due to developmental and considerable size differences of the larvae over time. Significant up-regulation of *interleukin 8b* (il8b) (Figs. 3-3C, 3-6C), *immunoglobulin heavy chain a* (*igha*), *ighb* and *immunoglobulin mu heavy chain c* (*ighmc*) (Fig. 3-4B, C, D; 3-7B, C, D), *chemokines* (*ccl19* and *ccl20*) (Figs. 3-4F, G and 3-7F, G), *cluster of differentiation 8 alpha* (*cd8a*) and *HLA class II histocompatibility antigen gamma chain* (*cd74*) (Figs. 3-4M, N and 3-7M, N), *interferon-gamma* (*ifng*) (Figs. 3-5B and 3-8B), and *ATP dependent RNA helicase lgp2* (*lgp2*) (Figs. 3-5E and 3-8E) occurred at 2 wpi. Significant upregulation of *interleukin 10* (*il10*) occurred at 4 wpi (Figs. 3-3E and 3-6E). Significant downregulation of *lymphocyte antigen 6 family member G6F* (*ly6g6f*) (Figs. 3-5A and 3-8A) and *ccl20* (Figs. 3-4G, 3-7G) occurred at 2 and 4 wpi, respectively. There were no significant differences in the expression levels of the remaining transcripts at either time point.

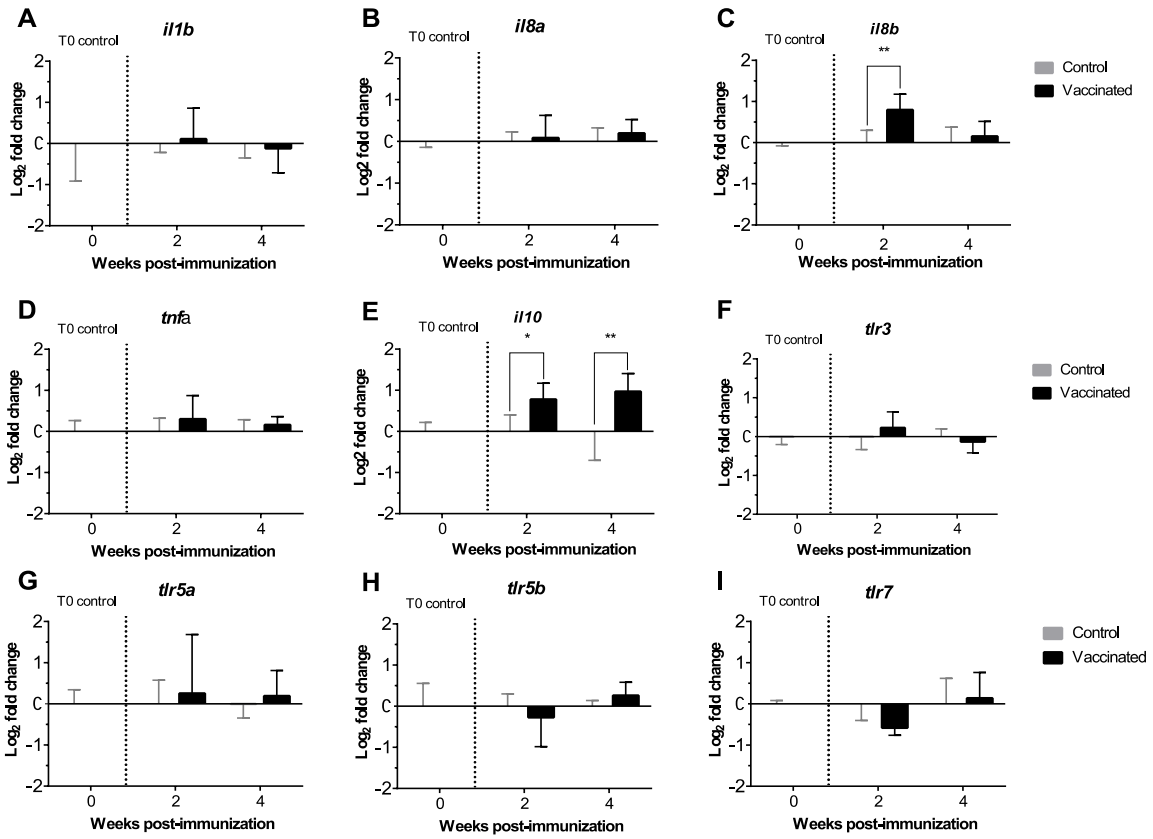

Figure 3-3. Transcript expression levels of cytokines and toll-like receptors in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii. (A–E). Cytokines; (F–I). Toll-like receptors. Transcript expression levels were assessed pre-immunization (T0 control, n = 3 individual pools of 10 larvae each), 2 wpi (n = 3 pools of 10 larvae each), and 4 wpi (n = 3 pools of 5 larvae each). Time point controls post-mock immunization were collected in a similar fashion at 2 and 4 wpi. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and normalized using $\log_2$; *etif3d* and *rpl32* were used as endogenous controls. A two-way ANOVA test, followed by the Sidak multiple comparisons post hoc test was used to assess significant differences between the treatments (control and vaccinated) at each individual time point. Asterisks (*) represent significant differences (* $p < 0.05$, ** $p < 0.01$)

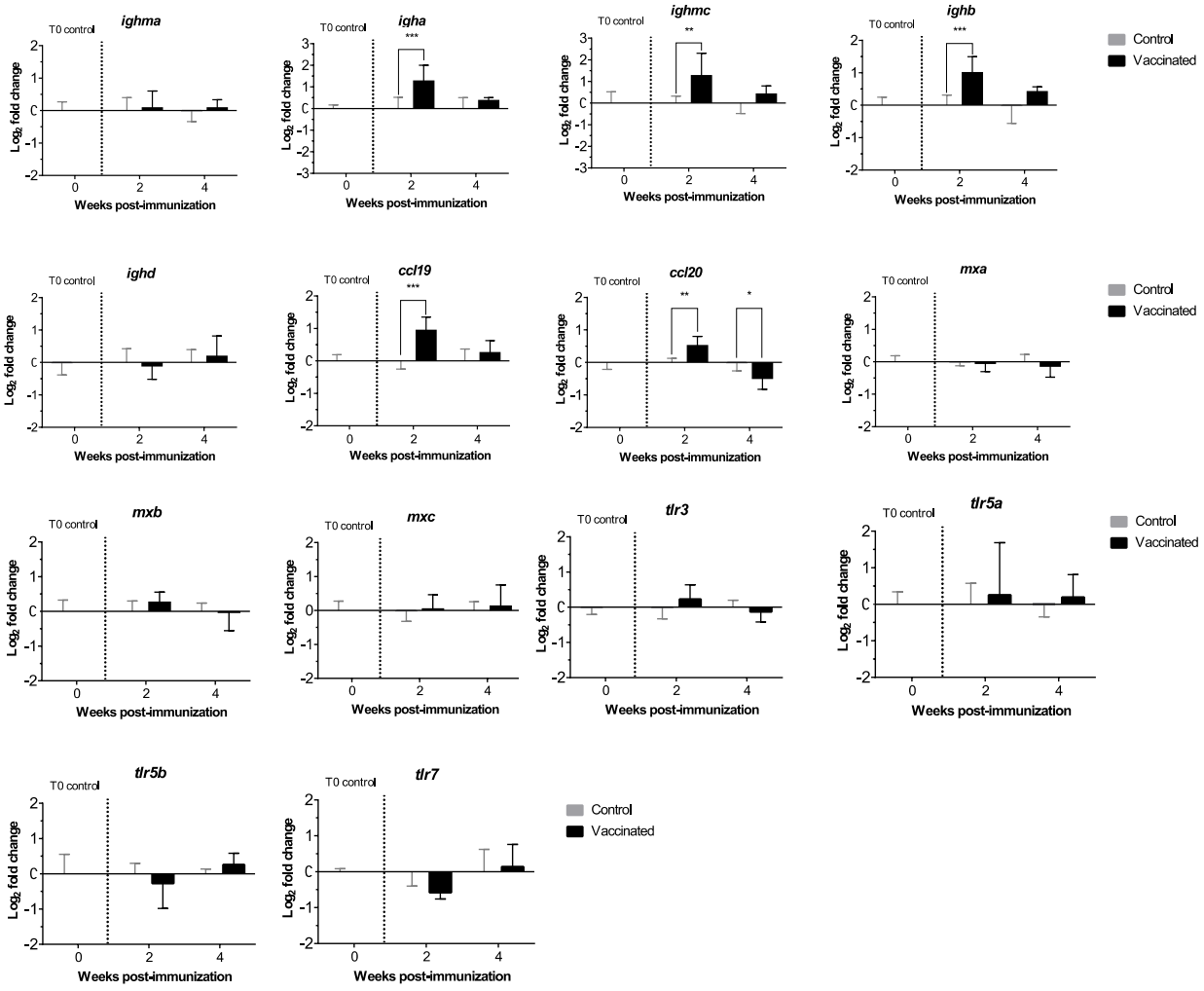

Figure 3-4. Transcript expression levels of immunoglobulin heavy locus genes, cytokine CC genes, and interferon-induced GTP-binding proteins genes, and the cluster of differentiation genes in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii (A–E). Immunoglobulin heavy locus genes (F–J). Cytokine CC genes and interferon-induced GTP-binding proteins genes (K–N). The cluster of differentiation genes. Transcript expression levels were assessed pre-immunization (T0 control, n = 3 individual pools of 10 larvae each), 2 weeks post-immunization (n = 3 pools

of 10 larvae each), and 4 weeks post-immunization (n = 3 pools of 5 larvae each). Time point controls post-mock immunization were collected in a similar fashion to those collected at weeks 2 and 4. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and normalized using $\log_2$; *etif3d* and *rpl32* were used as endogenous controls. A two-way ANOVA test followed by the Sidak multiple comparisons post hoc test were used to assess significant differences between the treatments (control and vaccinated) at each individual time point. Asterisks (*) represent significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

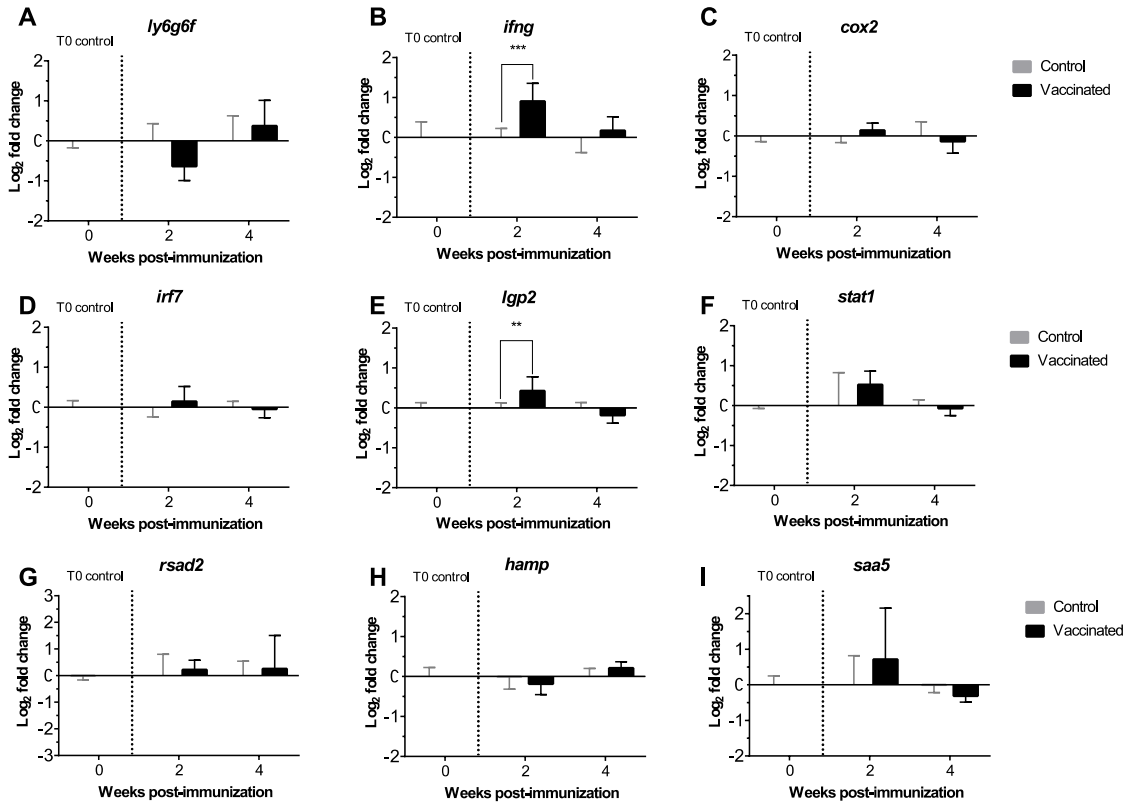

Figure 3-5. Transcript expression levels of other immune-related genes in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii (A–I). Transcript expression levels were assessed pre-immunization (T0 control, n = 3 individual pools of 10 larvae each), 2 weeks post-immunization (n = 3 pools of 10 larvae each), and 4 weeks post-immunization (n = 3 pools of 5 larvae each). Time point controls post-mock immunization were collected in a similar fashion to those collected at weeks 2 and 4. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and normalized using $\log_2$; *etif3d* and *rpl32* were used as endogenous controls. A two-way ANOVA test followed by the Sidak multiple comparisons post hoc test were used to assess significant differences between the treatments (control and vaccinated) at each individual time point. me point. Asterisks (*) represent significant differences (** $p< 0.01$, ***$p< 0.001$)

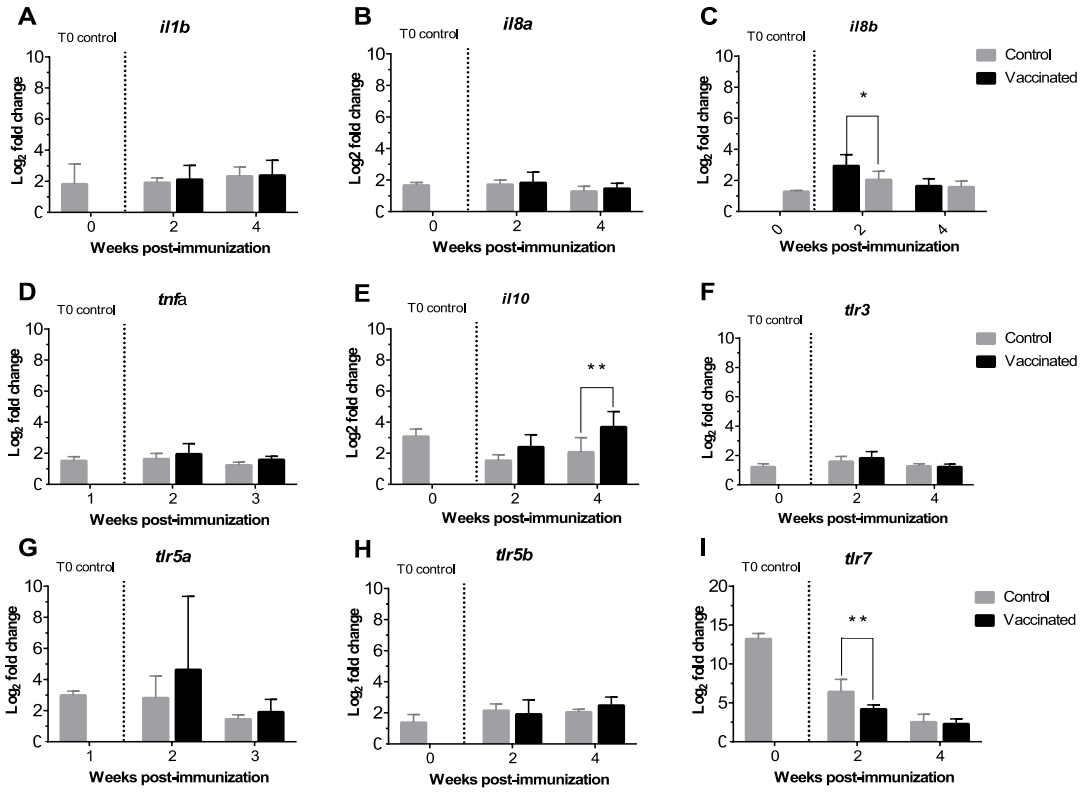

Figure 3-6. Transcript expression levels of cytokines and toll-like receptors in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii (A–E). Cytokines; (F–I). Toll-like receptors. Transcript expression levels were assessed pre-immunization (T0 control, n = 3 individual pools of 10 larvae each), 2 wpi (n = 3 pools of 10 larvae each), and 4 wpi (n = 3 pools of 5 larvae each). Time point controls post-mock immunization were collected in a similar fashion at 2 and 4 wpi. Relative expression was calculated using the RQ method and normalized using $\log_2$; *etif3d* and *rpl32* were used as endogenous controls. A two-way ANOVA test, followed by the Sidak multiple comparisons post hoc test was used to assess significant differences between the treatments (control and vaccinated) at each individual time point. Asterisks (*) represent significant differences (* $p < 0.05$, ** $p < 0.01$)

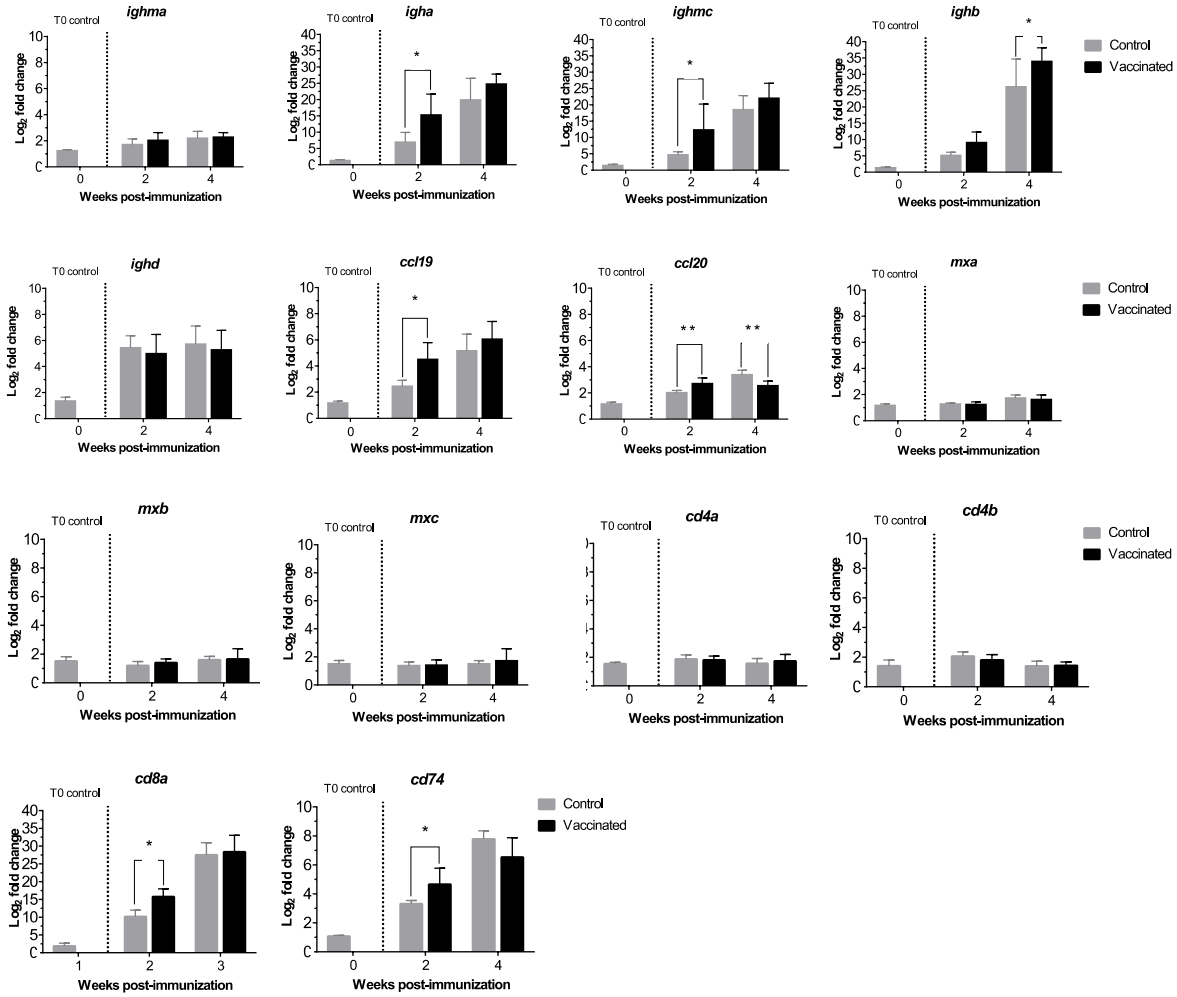

Figure 3-7. Transcript expression levels of immunoglobulin heavy locus genes, cytokine CC genes, and interferon-induced GTP-binding proteins genes, and the cluster of differentiation genes in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii (A–E). Immunoglobulin heavy locus genes (F–J). Cytokine CC genes and interferon-induced GTP-binding proteins genes (K–N). The cluster of differentiation genes. Transcript expression levels were assessed pre-immunization (T0 control, n = 3 individual pools of 10 larvae each), 2 weeks post-immunization (n = 3 pools of 10 larvae each), and 4 weeks post-immunization (n = 3 pools of 5 larvae each). Time

point controls post-mock immunization were collected in a similar fashion to those collected at weeks 2 and 4. Relative expression was calculated using the RQ method and normalized using $\log_2$; *etif3d* and *rpl32* were used as endogenous controls. A two-way ANOVA test followed by the Sidak multiple comparisons post hoc test were used to assess significant differences between the treatments (control and vaccinated) at each individual time point. Asterisks (*) represent significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

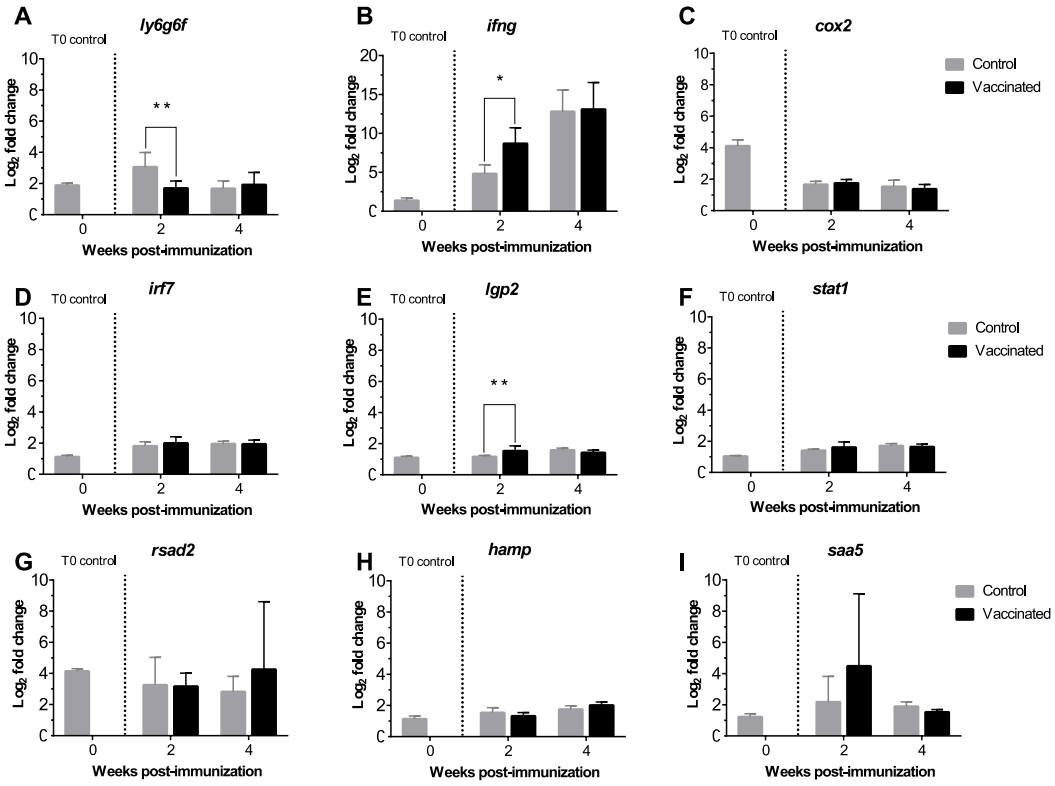

Figure 3-8. Transcript expression levels of other immune-related genes in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii (A–I). Transcript expression levels were assessed pre-immunization (T0 control, n = 3 individual pools of 10 larvae each), 2 weeks post-immunization (n = 3 pools of 10 larvae each), and 4 weeks post-immunization (n = 3 pools of 5 larvae each). Time point controls post-mock immunization were collected in a similar fashion to those collected at weeks 2 and 4. Relative expression was calculated using the RQ method and normalized using log_2; *etif3d* and *rpl32* were used as endogenous controls. A two-way ANOVA test followed by the Sidak multiple comparisons post hoc test were used to assess significant differences between the treatments (control and vaccinated) at each individual time point. Asterisks (*) represent significant differences (** $p< 0.01$, ***$p< 0.001$)

3.3. Vaccine challenge

Immunized lumpfish were challenged at 45 wpi with 7 times the LD50 dose for *V. anguillarum* J360 (7.8×10^5 CFU dose^{-1}) to determine the effectiveness of the vaccine (Fig. 2-5). Mortality in lumpfish who had been orally mock immunized as larvae and then mock-orally boosted as juveniles started at 3 days post-challenge, with 100% mortality by day 10 post-challenge. Mortality in lumpfish that had been orally immunized as larvae and then orally boosted as juveniles started at 3 days post-challenge, with a final RPS of 2% (Fig. 3-9A). Mortality in lumpfish that had been mock orally immunized as larvae and then both mock orally and i.p. boosted as juveniles started at 7 days post-challenge, with 100% mortality by 20 days post-challenge (Fig. 3-9B). Lumpfish that had been orally immunized as larvae and then both orally and i.p. boosted as juveniles survived the i.p. challenge with *V. anguillarum*, with a RPS of 76.5% ($p < 0.0001$) (Fig. 3-9B).

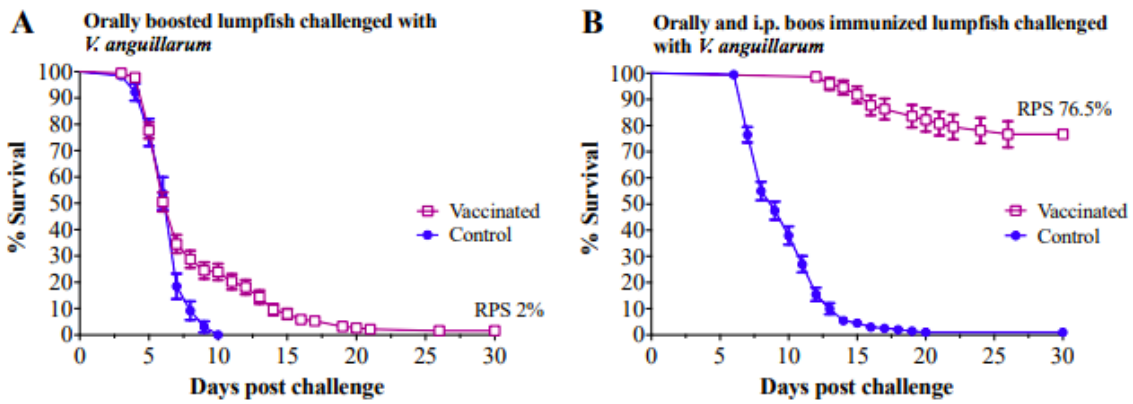

Figure 3-9. Cumulative survival rate of orally and i.p. immunized lumpfish after i.p. challenge with *V. anguillarum* (7.8×10^5 CFU dose^{-1}). (A) Survival (%) of orally immunized and orally boosted lumpfish after *V. anguillarum* challenge. Lumpfish were

orally immunized as larvae and then orally boosted as juveniles. Control groups were mock-vaccinated using the same inoculation route. After 45 weeks post-initial immunization, the animals were then i.p. challenged; each treatment consisted of two tanks (see Fig. 2-5). (B) Survival of orally immunized and i.p. boosted lumpfish. Lumpfish were orally immunized as larvae and then orally boosted as juveniles (see Fig. 2-5). Control groups were mock-vaccinated using the same inoculation route. After 45 weeks post-initial immunization, the animals were then i.p. challenged; Each treatment consisted of two tanks (see Fig. 2-5). Survival was assessed for 30 days. RPS: relative percentage survival; $p < 0.0001$.

4. DISCUSSION AND CONCLUSION

4.1. Discussion

Vibriosis is one of the most common bacterial diseases affecting lumpfish aquaculture (Vasquez, 2020a). As mentioned previously, immunization of fish at an early age with a needle-free vaccine, and with minimal stress during immunization is the ideal vaccine delivery method for finfish aquaculture (Plant, 2011). However, the effectiveness of bath and oral vaccine delivery to small fish or larvae has been evaluated with varied results (Villumsen, 2014). Commercial bath vaccines have been used in lumpfish with low effectiveness against local *V. anguillarum* isolates (Chakraborty,2019). However, the efficacy of an orally administered vaccine in lumpfish at the early-life stages remains unknown. Here, we evaluated the efficacy of an orally delivery *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii in lumpfish larvae. Approximately 9 months after the initial oral vaccination, lumpfish were either orally or both orally and i.p. boosted, and the effectiveness of the vaccines was evaluated by assessing the RPS after a lethal i.p. *V. anguillarum* challenge.

First, we evaluated the *V. anguillarum* bacterin uptake in *A. salina* nauplii and, thereafter this bio-encapsulated bacterin, in the gut of lumpfish larvae. Our observations indicated that the *V. anguillarum* bacterin was fully bio-encapsulated by the *A. salina* nauplii after 3 h and was maintained for at least 6 d at 6 $^{\circ}$C (Fig. 3-1). Similar results were observed by Campbell et al., (1993), where the *V. anguillarum* bacterin showed maximum bio-encapsulation after 1 h or 2 h using $1.5x10^{7}$ CFU mL^{-1} or $1.5x10^{6}$ cells mL^{-1}, respectively. Vaccine bio-encapsulation in *A. salina* nauplii protects the antigens from the

intestinal tract of the fish and facilitates the recognition of antigens by macrophages in the mucosal layer of the hindgut (Lin, 2005). The effectiveness of protecting the antigen from gastrointestinal digestion and its delivery to the hindgut of fish larvae has been demonstrated in previous studies (Lin, 2005; 2007). Here, we confirmed the presence of the *V. anguillarum* bacterin in the *A. salina* nauplii and in the gut of fish larvae 6 h post-oral immunization (Fig. 3-2). These results validated the internalization of the *V. anguillarum* bacterin in the lumpfish gut.

The expression profiles of 32 TOIs related to innate and adaptive immunity were evaluated at 0, 2, and 4 wpi. In pre-immunized larvae, we did not see any expression of the TOIs, which was expected. When considering orally immunized compared to larvae who had been orally mock immunized at 2 and 4 wpi, there were no significant differences in levels of pro-inflammatory cytokines (*tnfa*, *il1b*, *il8a*; Fig. 3-3A, B, D; Figs. 3-6A, B, D), toll-like receptors (*tlr3*, *tlr5a*, *tlr5b*; Figs. 3-3F, G, H; Figs. 3-6F, G, H), immunoglobulin heavy chain transcripts (*ighma*, *ighd*; Figs. 3-4A, E; Figs. 3-7A), interferon-induced effectors (*mxa*, *mxb*, *mxc*; Figs. 3-4H, I, J; Figs. 3-7H, I, J), cluster of differentiation transcripts (*cd4a*, *cd4b*; Figs. 3-4K, L; Figs. 3-7K, L) and other immune-related transcripts (*cox2*, *irf7*, *lgp2*, *stat1*, *rsad2*, *hamp*, *saa5*; Figs. 3-5 and 3-8) Figs. 3-5 and 3-8) at either 2 or 4 wpi. In contrast, levels of *il8b*, *igha*, *ighmc*, *ighb*, *ccl19*, *ccl20*, *cd8a*, *cd74*, *infg* and *lgp2* were significantly up-regulated, and levels of *ly6g6f* and *tlr7* were significantly down-regulated at 2 wpi (Figs. 3-3 to 3-5; Figs.3-6 to 3-8). Levels of *il10* were significantly up-regulated and levels of *ccl20* were significantly down-regulated at 4 wpi (Figs. 3-3E, 3-4G; Figs. 3-6E, 3-7G). These results indicate that 35 d old lumpfish larvae are not highly

immune stimulated by oral immunization, suggesting that the interaction between the lymphoid tissues and the vaccine was not enough to trigger adaptive immune protection.

il8 and *infg* play important roles in the recruitment of monocytes and neutrophils to sites of inflammation. Whereas *il10* acts as anti-inflammatory cytokine and, as such, plays a crucial role in the regulation of the inflammatory response (Min, 2001). Although there are studies on the expression of *il8* and *il10* in fish, the role of these interleukins in early developmental stages of lumpfish is still unknown. In the current study, in orally immunized larvae compared to mock-orally immunized larvae, *il8* and *infg* were significantly up-regulated at 2 wpi, and *il10* significantly up-regulated at 4 wpi (Figs. 3-3C, E, 3-5B; Figs. 3-6C, E, 3-8B). Similar expression profiles for *infg* and *il10* have been observed in Atlantic salmon that had been infected with the salmonid alphavirus subtype-3 (SAV-3) (Xu, 2012) or immunized with the *A. salmonicida* vaccine (Kumari, 2013). *lgp2* is a member of the RLR family, which participates in the recognition of viral RNA pathogen-associated molecular patterns (PAMPs) in the cytoplasm and induces the synthesis of *infg* (Ohtani, 2010; Chang, 2011; Van der Veen, 2018; Zhang, 2018). Similarly, our results showed that *lgp*2 and *infg* were significantly up-regulated at 2 wpi (Figs. 3-5B, E; Figs. 3-8B, E). The results suggest that the oral immunization of lumpfish larvae triggers an innate immune response that is later regulated via the canonical anti-inflammatory cytokine *il10*.

TLRs play an important role in early innate and adaptive immunity by detecting PAMPs in bacteria and viruses (Arancibia, 2007; Jayaramu, 2017; Ji, 2018). TLRs activate the transcription factor NF-κB, resulting in the production of several pro-inflammatory cytokines such as *il1b*, *tnfa*, *il8*, *il10*, *il6*, *il12*, *il17*, *infg*, and tumour necrosis factor (*tnf*)

(Barton, 2002; Eggestol, 2018). Expression levels of *tlr3*, *tlr5* and *tlr7* were not significantly different in orally immunized compared to mock-orally immunized larvae at either time point (Fig. 3-3F, G, H, I), however *tlr*7 was significantly down-regulated at 2 wpi in RQ statistical analysis (Fig. 3-6I). These results suggest that the vaccine does not induce a full immune response in larvae.

ly6g6f is a member of the superfamily lymphocyte antigen-6 (Ly6)/urokinase-type plasminogen activator receptor (uPAR) (Upadhyay, 2019). Ly6/uPAR proteins have functions in cell proliferation, migration, cell-cell interaction, immune cell maturation, macrophage activation, T lymphocyte development, differentiation, and cytokine production (MacNeil, 1993; Mallya, 2006; Loughner, 2016). The function of *ly6g6f* in fish is not yet defined and, in this study, the expression of *ly6g6f* was significantly down-regulated at 2 wpi in lumpfish larvae (Figs. 3-5A, 3-8A). These results agree with the low level of immune protection.

The *igh* (immunoglobulin heavy locus) encodes the IgM heavy chains and these loci have been characterized in several fish species, including fugu, rainbow trout, zebrafish, and Atlantic salmon (Danilova, 2005; Savan, 2005; Yasuike, 2010). It has been established that *igh* plays a role during the adaptive immune response by recognizing foreign antigens for phagocytosis, and the complement system (Schroeder, 2010). Here, we found that *igha*, *ighd*, and *ighmc* expression were significantly upregulated in orally immunized compared to larvae who had been orally mock immunized at 2 wpi (Figs. 3-4B, C, E; and 3-7B, C, E). These results suggest the oral immunization of larvae triggers some level of an adaptive immune response, but it seems insufficient to trigger memory immune protection.

ccl19 is a chemokine known to orchestrate the migration of dendritic cells (DCs) and T cells into lymphoid tissue or vaccination sites, and is also involved in immune tolerance and inflammatory responses (Bromley, 2008; Yan, 2019). *ccl20* attracts lymphocytes and DCs towards epithelial cells to mucosal immune sites under inflammatory conditions early in an immune response (Liu, 2020). In orally immunized compared to larvae who had been orally mock immunized, there was a significant increase in levels of *ccl19* and *ccl20* at 2 wpi, while there was a significant decrease in *ccl20* levels at 4 wpi (Figs. 3-4F, G and 3-7F, G). These results aligned with the expression patterns of other transcripts evaluated here, supporting the idea that lumpfish larvae did initiate and adaptive immune response to the oral immunization.

CD4 (a classical marker of T helper cells) and CD8 (a marker of cytotoxic lymphocytes) are polypeptides playing an important role in signal transduction, and activation of T-helper cells and cytotoxic T cells, respectively (Buonocore, 2006). We found that *cd8* was significantly up-regulated in orally immunized compared to larvae who had been orally mock immunized at 2 wpi (Figs. 3-4M, 3-7M). However, *cd4* was not significantly dysregulated. These results suggest that CD8 cellular-mediated adaptive immunity, but not the CD4 response, was activated in lumpfish larvae aligning with the lack of immune protection triggered by the oral immunization.

CD74 is the MHC class II-associated invariant chain, which plays a role in antigen presentation (Moldenhauer, 1999; Beswick, 2009). CD4 and CD74 lost their original functions in anglerfish (*Lophius piscatorius*) and Atlantic cod (*Gadus morhua*) (Star, 2011; Trowsdale, 2013; Dijkstra, 2018; Dubin, 2019). Here, we found that these transcripts are present in lumpfish, and although *cd74* was upregulated in orally immunized compared to

larvae who had been orally mock immunized at 2 wpi, *cd4a* and *cd4b* were not (Fig. 3-4K, L, N; Figs. 3-7K, L, N). These results revealed that oral immunization in lumpfish larvae triggers a partial adaptive immune response.

The lumpfish larvae were vaccinated after the yolk sac was absorbed, after which the larvae exhibited an active feeding behavior. Although there is no literature about the immunity of lumpfish larvae, it is well known that lumpfish larvae are more mature and active than other marine fish (Brown, 1986). It has also been shown that the main immune organs of lumpfish develop after hatching (Imsland, 2019). These reports, in addition to our current results, suggest that lumpfish larvae are immune competent, and antigens need to be delivered across the epithelia to trigger full immunity. The transcript expression levels (Figs 3-3 to 3-8) also indicated that lumpfish larvae are immune stimulated by oral immunization, but not enough to trigger immune protection. For instance, the expression of *il8b*, *il0*, *igha*, *ighmc*, *ighb*, *cd8*, and *cd74* was upregulated in orally immunized larvae (Figs. 3-3, 3-4; Figs. 3-6, 3-7). It seems that oral immunization with *V. anguillarum* bacterin in lumpfish larvae triggered Th1-like immune response and cellular immunity, which is related to *il10* and *cd8* upregulation. This is the first study on lumpfish larvae molecular immunity and provides novel knowledge and a baseline to study the ontogeny of the immune system in lumpfish.

The effectiveness of vaccination in fish depends on the delivery, vaccine design, and the fish species. For instance, mortality in lumpfish bath immunized and i.p. boosted with a commercial polyvalent formalin-inactivated *V. anguillarum* O1 and O2 vaccine was only delayed in an i.p. challenge using *V. anguillarum* (Chakraborty, 2019). Similar to our current results, a commercial bivalent whole-cell *V. anguillarum* O1 and O2 vaccine

delivered by immersion and followed by an i.p. boost immunization in European sea bass (*Dicentrarchus labrax*) conferred approximately 99% survival against a *V. anguillarum* i.p. challenge (Galeotti, 2013). In this study, we observed that lumpfish orally immunized as larvae and then orally boosted as juveniles did not survive the *V. anguillarum* i.p. challenge (Figure 3-9A). Nevertheless, we determined that oral vaccination delayed mortality in lumpfish challenged with *V. anguillarum*, suggesting that the oral vaccination did stimulate fish immunity, but not enough to confer protection. Similar results were found in salmonids orally immunized against *Yersinia* and *V. anguillarum*, where oral immunization conferred no or low immunity to juvenile immunized fish (Johnson, 1983a, b, c; Chettri, 2015). In contrast, lumpfish orally immunized as larvae and then both orally and i.p. boosted as juveniles showed a significant RPS (76.5%) to the *V. anguillarum* i.p. challenge challenge (Figure 3-9B). This suggests that the orally administered vaccines were not reaching the deep lymphoid tissues, either in the larvae or juvenile fish, and as such, oral immunization was not effective in contrast to the i.p. delivered vaccine. Therefore, it is suggested that inactivated *V. anguillarum* vaccines for lumpfish should be administered using the i.p. route to confer acceptable levels of immune protection.

4.2. Conclusions

Oral immunization of lumpfish larvae using bio-encapsulated bacterin demonstrated that it reached the larval gut and stimulated an immune response by increasing innate immunity. However, oral immunization did not trigger an evident adaptive immune response, even after oral boost immunization. *V. anguillarum* bacterin that had been orally administered delayed mortality and did not confer protection against

the i.p. *V. anguillarum*. In contrast, i.p. immunization conferred significant immune protection. These results suggest the need for oral vaccines that have the capability of crossing the epithelium and reaching the deep lymphoid tissues to trigger immune protection.

5. SUMMARY

The development of effective oral vaccines to overcome the need for injection is essential for vaccines applied in aquaculture. In this study, *V. anguillarum* bacterin delivered as an oral vaccine demonstrated a delay in mean time to death after ip challenge but was not effective in lumpfish, which is in contrast to the i.p. vaccine delivery method that protects lumpfish against vibriosis. This suggests that orally administered vaccines were not reaching deep lymphoid tissues, either in the larvae or juvenile fish, and as such, oral immunization was not effective. Oral vaccines that have the capability of crossing the epithelium and reaching deep lymphoid tissues are needed to confer an effective protection to the fish. Although high levels of protection were not observed by oral vaccine against *V. anguillarum* in the current study, novel information has been gained regarding the immune response of lumpfish larvae during early immunization. Additionally, an i.p. injection method was deemed to be the most efficient method for stimulating a protective immune response in cultured juvenile lumpfish against *V. anguillarum.*

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7. APPENDICES

Appendix I. Ct values of transcript expression levels of immune-related genes in lumpfish (*Cyclopterus lumpus*) larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii.

Sample	Study 1								Study 2							
	ccl19	***cox2***	***hamp***	***il8a***	***il8b***	***il10***	***etif3d***	***rpl32***	***lgp2***	***mxa***	***mxb***	***mxc***	***stat1***	***tlr3***	***etif3d***	***rpl32***
W0_C1_F1	28.97	27.42	25.01	26.89	28.98	30.40	22.12	21.12	28.80	27.11	29.48	29.67	26.77	29.29	22.26	21.98
W0_C1_F1	29.06	27.22	25.12	26.96	28.98	29.94	22.18	20.87	28.86	27.34	29.61	29.74	26.69	29.11	22.35	22.00
W0_C1_F1	28.93	27.27	25.18	26.98	29.00	30.21	22.03	20.89	29.04	27.43	29.49	29.56	26.62	29.16	22.32	22.01
W0_C1_F2	29.08	27.25	25.60	27.26	29.13	30.78	22.20	20.91	28.72	27.25	29.09	29.55	27.03	29.95	22.46	22.15
W0_C1_F2	29.18	27.11	25.66	27.17	29.34	30.26	22.20	21.11	29.00	27.31	29.24	29.40	27.06	29.25	22.47	22.51
W0_C1_F2	29.16	27.11	25.53	27.49	29.20	30.67	22.23	21.13	29.00	27.11	29.13	29.20	26.96	29.30	22.50	22.13
W0_C1_F3	29.72	27.40	25.71	27.32	29.07	31.14	22.21	21.20	29.10	27.05	28.99	29.24	26.99	29.65	22.44	22.29
W0_C1_F3	29.37	27.41	25.63	27.30	29.25	30.75	22.20	21.25	29.00	27.04	29.18	29.50	27.01	29.89	22.39	22.23
W0_C1_F3	29.35	27.44	25.58	27.23	29.09	30.34	22.17	20.98	29.19	27.28	29.26	29.35	26.99	29.70	22.39	22.20
W2_C1_F1	28.04	28.49	24.50	26.88	27.78	31.17	21.98	20.87	28.59	26.97	29.83	29.56	26.19	28.72	22.22	22.00
W2_C1_F1	28.21	28.43	24.62	27.08	30.24	30.81	21.95	20.97	28.80	26.99	29.59	29.62	26.20	28.69	22.28	21.95
W2_C1_F1	28.16	28.24	24.63	26.99	27.83	31.48	22.05	21.04	28.61	26.92	29.80	29.42	26.24	28.64	22.26	22.03
W2_C1_F2	27.89	28.31	24.52	26.85	28.36	31.35	21.89	20.89	28.63	26.59	28.95	29.22	26.08	28.47	22.06	22.05
W2_C1_F2	27.88	28.32	24.51	26.80	29.75	30.90	21.89	20.81	28.43	26.56	28.58	28.85	26.02	28.48	22.10	21.77
W2_C1_F2	27.98	28.33	24.49	26.92	28.37	31.62	21.88	20.65	28.45	26.80	28.70	28.91	25.94	28.43	21.99	21.71
W2_C1_F3	27.49	27.96	24.48	26.67	28.04	30.93	21.87	20.78	28.58	26.86	29.15	29.21	26.12	28.31	22.19	21.93

Sample	Study 1								Study 2							
	ccl19	***cox2***	***hamp***	***il8a***	***il8b***	***il10***	***etif3d***	***rpl32***	***lgp2***	***mxa***	***mxb***	***mxc***	***stat1***	***tlr3***	***etif3d***	***rpl32***
W2_C1_F3	27.22	28.32	24.43	26.54	27.79	31.53	21.86	20.71	28.60	26.79	29.45	29.52	26.26	28.59	22.20	21.97
W2_C1_F3	27.25	27.97	24.45	26.57	27.75	30.92	21.95	20.66	28.67	26.81	29.61	29.29	26.22	28.26	22.22	21.98
W2_C2_F1	28.19	28.79	25.46	27.54	28.65	33.40	22.07	21.14	29.18	27.03	29.84	29.98	26.38	29.46	22.42	22.51
W2_C2_F1	28.06	28.91	25.46	27.62	28.71	31.27	22.13	21.16	28.81	27.18	29.91	29.96	26.40	29.46	22.41	22.41
W2_C2_F1	28.11	28.73	25.52	27.48	28.78	31.35	22.03	21.14	28.94	27.01	30.17	30.02	26.37	29.40	22.37	22.32
W2_C2_F2	28.29	28.51	25.03	27.08	28.59	31.52	21.98	20.94	28.76	26.90	29.39	28.97	32.13	28.84	22.21	21.98
W2_C2_F2	28.04	28.46	25.04	27.26	28.81	32.30	21.96	21.01	28.66	26.78	29.57	28.96	26.17	29.15	22.14	21.97
W2_C2_F2	27.75	28.62	24.92	27.12	28.58	31.84	22.02	20.87	28.32	26.96	29.30	29.28	26.31	29.02	22.25	21.96
W2_C2_F3	28.10	28.75	25.00	26.84	28.66	31.98	21.84	21.02	28.87	26.96	29.48	29.84	26.33	29.11	22.04	21.98
W2_C2_F3	28.22	28.53	25.07	26.91	28.53	31.77	21.92	20.98	28.83	27.12	29.42	30.16	26.35	29.25	22.08	21.98
W2_C2_F3	28.21	28.69	25.22	26.89	28.55	32.72	21.93	21.12	28.90	27.02	29.78	29.51	26.38	29.11	22.03	22.02
W2_V1_F1	26.94	28.76	25.21	26.53	27.89	31.06	22.41	21.25	28.02	26.75	29.07	29.06	25.72	28.71	22.51	22.19
W2_V1_F1	26.81	28.36	25.06	26.68	27.89	30.55	22.36	21.34	28.11	26.79	29.01	29.10	25.69	28.68	22.49	22.18
W2_V1_F1	26.68	28.54	25.09	26.53	27.76	30.67	22.38	21.17	28.00	26.83	28.99	29.16	25.88	28.64	22.54	22.16
W2_V1_F2	27.14	28.80	25.62	27.12	28.06	30.50	22.49	21.50	28.56	27.31	29.44	29.63	26.40	28.79	22.76	22.61
W2_V1_F2	27.21	28.79	25.56	27.20	27.99	30.84	22.57	21.48	28.64	27.21	29.80	29.62	26.27	28.74	22.81	22.54
W2_V1_F2	27.22	28.74	25.56	27.07	27.92	31.83	22.54	21.50	28.45	27.39	29.72	29.79	26.35	28.72	22.75	22.43
W2_V1_F3	27.76	28.93	26.03	27.67	28.04	31.73	22.60	21.43	29.16	27.75	29.55	30.36	26.70	29.00	22.80	22.57
W2_V1_F3	27.67	28.98	26.12	27.66	27.78	32.00	22.54	21.70	28.86	27.56	30.08	30.33	26.66	28.96	22.75	22.68
W2_V1_F3	27.73	29.28	25.99	27.62	28.17	31.34	22.61	21.49	28.88	27.61	29.99	30.32	26.74	29.17	22.81	22.67
W2_V2_F1	27.46	28.48	25.09	27.07	28.43	30.79	22.48	21.37	28.57	27.49	29.37	29.59	26.50	28.79	22.69	22.51
W2_V2_F1	27.63	28.68	25.29	27.10	28.59	31.37	22.47	21.37	28.90	27.40	29.87	29.93	26.57	28.94	22.77	22.34

Sample	Study 1								Study 2							
	ccl19	***cox2***	***hamp***	***il8a***	***il8b***	***il10***	***etif3d***	***rpl32***	***lgp2***	***mxa***	***mxb***	***mxc***	***stat1***	***tlr3***	***etif3d***	***rpl32***
W2_V2_F1	27.51	28.50	25.21	27.27	28.45	30.85	22.37	21.20	28.45	27.31	29.70	29.17	26.69	28.78	22.72	22.37
W2_V2_F2	27.96	28.91	25.74	28.43	28.31	31.76	22.45	21.39	28.97	27.48	29.73	30.44	26.55	29.54	22.76	22.62
W2_V2_F2	28.02	29.23	25.75	28.10	28.26	31.15	22.38	21.49	28.97	27.68	29.80	30.08	26.71	29.57	22.68	22.48
W2_V2_F2	28.05	28.97	25.61	27.99	28.30	31.76	22.44	21.29	29.09	27.66	29.60	30.35	26.61	29.55	22.72	22.44
W2_V2_F3	27.97	29.11	25.67	28.01	29.02	31.66	22.43	21.86	29.11	27.70	30.27	30.25	26.69	29.78	22.78	22.13
W2_V2_F3	28.05	29.14	25.87	28.07	29.03	31.96	22.50	21.93	29.18	27.67	29.94	30.37	26.91	29.55	22.85	22.25
W2_V2_F3	28.04	29.19	25.78	27.91	29.03	32.57	22.45	21.88	29.27	27.67	30.08	30.21	27.17	29.57	22.83	22.21
W4_C1_F1	28.66	30.08	26.08	28.94	30.73	32.23	23.33	22.79	29.45	27.95	29.97	30.42	27.02	30.11	23.53	22.82
W4_C1_F1	28.69	29.86	26.13	28.91	30.05	31.97	23.30	22.61	29.38	27.77	30.16	30.94	26.82	29.90	23.59	22.88
W4_C1_F1	28.69	30.20	26.12	28.96	30.72	32.73	23.33	22.73	29.71	27.72	30.31	30.60	26.98	30.42	23.56	22.85
W4_C1_F2	27.31	29.16	25.68	28.53	29.51	31.03	22.98	21.98	28.80	26.89	29.19	29.80	26.37	29.53	23.04	22.20
W4_C1_F2	27.31	29.12	25.68	28.75	29.52	31.32	22.97	22.00	28.51	26.81	29.56	29.71	26.57	29.64	23.13	22.47
W4_C1_F2	27.34	28.89	25.75	28.57	31.52	31.24	22.94	22.12	28.61	27.13	29.35	30.08	26.74	29.51	23.10	22.25
W4_C1_F3	28.17	29.17	25.50	28.40	No	31.75	23.00	22.26	28.76	26.93	29.85	29.56	26.81	29.92	23.08	22.36
W4_C1_F3	28.61	29.15	25.44	28.59	29.68	31.02	22.91	22.17	28.87	26.86	29.77	29.86	26.71	30.15	23.06	22.32
W4_C1_F3	28.19	29.27	25.48	28.49	29.82	31.38	22.94	22.27	29.00	26.91	29.40	30.07	26.71	30.01	23.06	22.26
W4_C2_F1	28.12	30.47	26.13	28.09	29.72	33.36	23.28	22.80	29.11	27.40	29.96	29.94	26.76	29.71	23.41	22.87
W4_C2_F1	27.96	30.13	25.72	28.20	29.94	33.41	23.27	22.59	29.22	27.21	30.05	30.04	26.99	29.96	23.37	22.80
W4_C2_F1	27.98	30.29	25.77	28.13	30.12	32.78	23.21	22.63	29.38	27.41	29.78	29.97	26.97	30.45	23.39	22.85
W4_C2_F2	27.96	30.01	25.67	28.53	29.92	34.01	23.04	22.25	28.92	26.73	29.18	29.80	26.68	29.88	23.22	22.52
W4_C2_F2	28.03	29.68	25.65	28.53	29.64	32.51	22.97	22.32	28.78	26.98	29.66	29.88	26.55	29.68	23.25	22.50
W4_C2_F2	28.03	29.89	25.55	28.46	29.86	32.11	22.99	22.21	28.90	26.80	29.47	29.64	26.60	29.65	23.21	22.51
W4_C2_F3	27.83	30.01	26.17	28.90	29.35	32.73	23.20	22.42	28.98	27.59	30.29	30.78	26.54	30.42	23.45	22.51

Sample	Study 1								Study 2							
	ccl19	***cox2***	***hamp***	***il8a***	***il8b***	***il10***	***etif3d***	***rpl32***	***lgp2***	***mxa***	***mxb***	***mxc***	***stat1***	***tlr3***	***etif3d***	***rpl32***
W4_C2_F3	27.79	29.97	26.17	28.83	29.59	32.61	23.14	22.39	28.98	27.47	30.11	30.37	26.76	29.91	23.38	22.57
W4_C2_F3	27.84	30.30	26.27	28.48	29.30	33.05	23.19	22.41	29.01	27.33	30.18	30.15	26.49	30.21	23.36	22.54
W4_V1_F1	28.02	30.15	25.91	28.86	30.80	31.53	23.42	22.94	29.61	27.70	29.97	30.78	27.13	30.86	23.68	22.96
W4_V1_F1	27.95	30.52	25.98	29.12	30.59	31.25	23.44	22.92	29.52	27.59	30.25	30.41	26.98	30.38	23.63	22.83
W4_V1_F1	27.51	30.18	25.89	28.95	30.82	31.53	23.44	22.86	29.17	27.63	30.45	30.03	27.05	30.23	23.67	22.95
W4_V1_F2	27.16	29.14	24.68	27.69	28.70	30.46	22.12	21.43	28.40	26.65	29.11	29.28	26.38	29.16	22.29	21.83
W4_V1_F2	27.19	28.97	24.73	27.84	28.63	29.93	22.14	21.51	28.55	26.80	29.37	29.72	26.18	29.05	22.29	21.86
W4_V1_F2	27.19	29.09	24.78	27.98	28.85	30.33	22.23	21.70	28.48	26.63	29.38	29.49	26.24	29.56	22.32	21.92
W4_V1_F3	28.02	30.25	26.13	28.84	29.97	32.00	23.15	22.73	29.56	27.72	30.32	30.88	27.07	30.12	23.39	22.93
W4_V1_F3	27.83	30.09	25.99	29.00	29.92	32.00	23.18	22.52	29.38	27.81	30.00	30.61	27.00	30.53	23.36	22.88
W4_V1_F3	27.83	30.32	26.02	28.73	30.06	31.30	23.13	22.77	29.85	27.82	30.69	30.72	27.10	30.55	23.33	22.73
W4_V2_F1	27.48	29.96	25.43	28.25	29.61	31.12	23.06	22.50	28.97	26.87	29.05	28.78	26.80	30.09	23.18	22.76
W4_V2_F1	27.46	30.53	25.44	27.90	29.71	31.21	23.04	22.61	29.16	26.78	28.59	28.83	26.92	30.07	23.24	22.69
W4_V2_F1	27.57	29.62	25.48	27.98	29.73	30.90	23.03	22.33	29.02	26.75	28.76	28.87	26.70	29.96	23.22	22.81
W4_V2_F2	28.85	30.36	25.97	28.56	30.60	31.98	23.34	22.88	29.77	27.90	30.25	30.31	26.80	31.14	23.35	22.98
W4_V2_F2	28.53	30.66	26.04	28.64	29.93	32.28	23.19	22.98	29.65	27.83	30.20	30.30	27.10	30.62	23.36	22.99
W4_V2_F2	28.80	30.83	26.03	28.66	30.44	33.02	23.24	22.82	29.52	28.04	30.45	30.76	27.12	30.36	23.35	22.94
W4_V2_F3	27.61	29.34	25.58	27.94	29.05	30.94	23.13	22.34	28.96	27.18	30.04	29.89	26.56	29.26	23.23	22.64
W4_V2_F3	27.68	29.54	25.65	28.04	29.43	31.39	23.12	22.59	28.97	27.10	29.97	29.99	26.60	29.52	23.27	22.45
W4_V2_F3	27.61	29.44	25.58	27.97	29.29	30.83	23.03	22.39	29.01	27.29	29.62	29.63	26.38	29.91	23.18	22.47
linker_plate1	27.48	28.02	24.94	26.87	28.19	30.48	22.07	20.87	28.44	26.84	29.46	29.14	25.96	28.79	22.35	22.15
linker_plate1	27.52	28.05	24.93	26.98	28.03	30.14	22.12	20.82	28.27	26.71	29.30	29.22	25.99	28.85	22.42	22.07
linker_plate1	27.46	28.12	24.86	26.86	28.05	30.30	22.05	20.92	28.44	26.90	29.21	29.10	26.00	28.84	22.36	22.14

Sample	Study 1								Study 2							
	ccl19	***cox2***	***hamp***	***il8a***	***il8b***	***il10***	***etif3d***	***rpl32***	***lgp2***	***mxa***	***mxb***	***mxc***	***stat1***	***tlr3***	***etif3d***	***rpl32***
linker_plate2	27.32	28.07	24.80	26.98	28.13	30.61	22.16	21.57	28.30	26.83	28.98	29.12	25.94	28.73	22.38	21.80
linker_plate2	27.38	28.10	24.99	27.06	28.27	30.22	22.05	21.31	28.34	26.73	29.06	29.18	25.93	28.62	22.30	21.68
linker_plate2	27.57	28.17	24.86	26.98	28.15	30.68	22.18	21.20	28.27	26.80	29.19	29.04	25.96	28.96	22.34	21.67

Sample	Study 3								Study 4							
	ccl20	*igha*	*ighb*	*irf7*	*cd74*	*saa5*	*etif3d*	*rpl32*	*cd4a*	*cd4b*	*il1b*	*tlr5a*	*tlr5b*	*tnfa*	*etif3d*	*rpl32*
W0_C1_F1	28.46	33.16	33.41	29.65	25.65	24.08	22.01	18.67	31.01	28.51	32.62	29.33	29.58	29.37	20.59	17.55
W0_C1_F1	28.63	33.32	32.30	29.55	25.50	24.06	22.03	18.70	30.87	28.75	31.97	29.51	29.46	29.22	20.67	17.58
W0_C1_F1	28.33	33.15	32.35	30.01	25.54	24.04	22.04	18.80	30.91	28.80	32.54	28.80	29.69	29.52	20.60	17.58
W0_C1_F2	28.85	31.91	32.81	29.56	25.80	23.99	22.08	18.83	30.92	29.47	30.72	30.34	30.94	28.96	20.65	17.55
W0_C1_F2	29.02	33.76	33.22	30.00	25.63	24.15	22.16	18.80	31.30	29.29	31.33	29.66	30.71	29.34	20.65	17.53
W0_C1_F2	29.08	33.56	33.34	29.80	25.62	24.02	22.10	18.77	30.65	29.58	31.08	29.66	30.26	28.89	20.67	17.51
W0_C1_F3	28.78	33.13	32.84	30.18	25.92	24.57	22.14	18.88	30.85	29.50	32.84	29.65	30.54	29.75	20.82	17.88
W0_C1_F3	28.76	32.92	32.44	30.09	25.74	24.55	22.08	18.87	30.94	29.50	32.94	29.97	30.44	29.86	20.77	17.77
W0_C1_F3	28.79	32.91	32.76	30.06	25.87	24.52	22.04	18.89	31.04	29.38	33.14	29.77	30.65	29.71	20.71	17.71
W2_C1_F1	27.48	30.73	30.08	28.96	23.81	23.48	21.95	18.61	30.44	28.50	32.01	30.20	28.99	29.42	20.63	17.55
W2_C1_F1	27.62	30.24	30.22	28.73	23.90	23.56	21.88	18.62	30.87	28.70	32.13	29.55	29.24	30.01	20.58	17.56
W2_C1_F1	27.63	30.46	31.10	28.80	23.93	23.50	21.84	18.64	31.01	28.55	32.07	30.37	29.37	29.29	20.60	17.55
W2_C1_F2	27.64	30.23	29.98	29.07	23.87	23.62	21.99	18.68	30.06	28.25	31.79	30.42	29.55	29.26	20.61	17.47
W2_C1_F2	27.60	29.94	30.76	28.81	23.83	23.46	21.92	18.65	30.30	28.44	31.75	30.06	29.47	29.13	20.62	17.43
W2_C1_F2	27.73	30.34	30.37	28.90	23.82	23.60	22.05	18.74	30.52	28.35	31.88	29.48	29.46	28.94	20.67	17.47
W2_C1_F3	27.43	29.79	30.18	28.55	23.74	23.99	21.86	18.62	30.19	28.13	31.40	29.48	29.07	28.82	20.54	17.36
W2_C1_F3	28.10	31.06	30.04	28.40	23.70	23.98	21.92	18.56	30.30	28.12	31.34	29.57	28.80	28.49	20.41	17.39
W2_C1_F3	27.38	29.49	30.36	28.51	23.78	23.97	21.91	18.63	30.39	28.19	31.30	29.26	28.62	28.62	20.57	17.35
W2_C2_F1	27.98	31.59	31.32	29.53	24.29	22.12	22.13	19.05	30.51	28.73	32.16	28.68	29.66	29.81	20.66	17.78
W2_C2_F1	28.29	31.61	31.15	29.44	24.23	22.04	22.05	18.98	30.68	30.55	31.95	28.82	29.74	29.44	20.55	17.73
W2_C2_F1	28.07	31.79	31.72	29.25	24.16	22.06	22.09	19.01	30.44	28.67	32.32	28.65	30.03	29.94	20.61	17.79
W2_C2_F2	28.09	30.72	31.06	29.45	24.28	24.30	22.31	19.17	30.91	28.53	31.83	29.98	29.11	29.29	20.43	17.50
W2_C2_F2	28.01	31.36	31.03	29.45	24.25	24.38	22.34	19.20	30.73	28.36	32.35	29.94	29.52	29.32	20.47	17.57

Sample	Study 3								Study 4							
	ccl20	*igha*	*ighb*	*irf7*	*cd74*	*saa5*	*etif3d*	*rpl32*	*cd4a*	*cd4b*	*il1b*	*tlr5a*	*tlr5b*	*tnfa*	*etif3d*	*rpl32*
W2_C2_F2	27.75	31.33	30.98	29.44	24.33	24.30	22.30	19.14	29.87	28.23	31.64	29.87	29.77	29.48	20.46	17.46
W2_C2_F3	27.98	31.25	30.35	29.29	24.06	23.91	21.96	18.74	30.38	28.85	32.00	29.91	29.63	29.47	20.45	17.46
W2_C2_F3	27.71	30.95	30.56	29.33	24.12	23.98	21.97	18.86	30.70	28.89	31.55	30.00	29.73	28.94	20.46	17.68
W2_C2_F3	27.95	31.58	31.30	29.28	24.23	23.92	22.03	18.88	30.12	28.94	31.88	30.18	29.62	28.83	20.48	17.67
W2_V1_F1	27.11	28.97	28.90	28.90	23.31	21.03	22.42	19.02	30.87	28.50	30.75	27.79	28.88	28.87	20.99	17.88
W2_V1_F1	27.21	29.27	29.42	28.93	23.30	21.03	22.44	18.96	30.68	28.97	31.41	27.69	29.01	28.67	21.07	17.85
W2_V1_F1	27.27	28.69	29.26	28.75	23.32	21.03	22.48	19.00	30.38	28.82	31.25	27.72	29.10	28.50	21.03	17.91
W2_V1_F2	27.42	29.42	29.57	28.98	23.61	21.58	22.43	19.01	31.43	28.97	31.66	28.35	29.59	29.08	20.98	17.84
W2_V1_F2	27.42	29.68	29.65	28.95	23.68	21.70	22.47	19.05	30.98	28.98	31.53	28.50	29.73	28.95	21.10	17.92
W2_V1_F2	27.45	29.48	30.01	29.12	23.50	21.71	22.41	19.01	31.14	28.83	31.44	28.42	29.82	28.70	20.99	17.87
W2_V1_F3	27.76	30.14	30.23	29.13	24.02	24.37	22.56	19.18	31.29	29.74	31.72	30.42	30.67	29.37	21.18	18.08
W2_V1_F3	27.59	30.32	30.34	29.36	24.10	24.40	22.44	19.18	31.38	29.31	32.19	30.60	30.34	29.51	21.20	18.05
W2_V1_F3	27.57	30.58	30.81	29.24	24.01	24.31	22.57	19.30	31.31	29.44	32.35	30.69	30.83	28.94	21.18	18.06
W2_V2_F1	27.84	29.39	29.99	29.25	23.78	24.37	22.43	19.03	31.44	28.83	33.05	30.38	29.71	29.20	20.98	18.10
W2_V2_F1	27.92	29.29	29.97	29.06	23.75	24.51	22.65	19.06	31.23	28.80	32.86	30.15	30.03	29.74	21.11	17.94
W2_V2_F1	27.79	29.33	30.13	29.08	23.86	24.52	22.66	19.01	31.14	29.00	32.50	30.51	29.96	29.46	21.06	17.91
W2_V2_F2	28.30	30.95	30.97	30.05	24.19	23.61	22.54	19.32	30.79	29.90	33.00	31.31	31.15	30.89	21.11	18.07
W2_V2_F2	28.30	31.55	30.64	29.87	24.08	23.68	22.42	19.23	30.86	29.52	32.80	32.00	31.15	30.29	21.12	18.03
W2_V2_F2	28.00	31.02	30.76	30.20	24.23	23.66	22.48	19.28	31.17	29.71	33.91	30.57	30.89	29.49	21.05	18.01
W2_V2_F3	27.91	30.23	30.53	29.50	24.47	24.40	22.52	19.34	30.76	29.11	32.91	31.11	30.58	29.84	21.07	18.11
W2_V2_F3	27.72	30.41	30.57	29.70	24.61	24.27	22.51	19.31	31.14	29.29	32.46	31.04	30.93	30.45	21.15	18.04
W2_V2_F3	27.82	30.22	29.96	29.73	24.47	24.33	22.51	19.33	31.34	29.39	32.42	31.44	30.73	29.96	21.14	18.08
W4_C1_F1	28.02	30.52	29.84	30.32	24.02	24.99	23.47	20.12	31.94	30.03	32.72	31.57	30.87	30.89	21.94	18.89

Sample	Study 3								Study 4							
	ccl20	***igha***	***ighb***	***irf7***	***cd74***	***saa5***	***etif3d***	***rpl32***	***cd4a***	***cd4b***	***il1b***	***tlr5a***	***tlr5b***	***tnfa***	***etif3d***	***rpl32***
W4_C1_F1	28.15	30.62	29.90	30.30	24.07	25.08	23.36	20.12	31.93	30.40	31.25	31.79	30.39	30.92	22.02	18.86
W4_C1_F1	28.20	30.71	29.56	30.43	23.95	25.04	23.40	20.07	32.11	30.29	33.20	31.46	30.70	30.10	22.03	18.87
W4_C1_F2	27.60	29.54	28.73	30.07	23.45	24.08	23.05	19.54	31.38	29.38	31.65	31.34	30.62	30.21	21.66	18.40
W4_C1_F2	27.63	29.21	28.70	29.86	23.41	24.00	23.04	19.62	31.57	29.56	31.89	31.33	30.36	30.15	21.66	18.44
W4_C1_F2	27.63	29.01	28.96	29.96	23.54	24.05	23.06	19.66	31.51	29.98	32.05	31.12	30.25	29.29	21.66	18.35
W4_C1_F3	27.74	29.53	28.88	30.17	23.48	24.30	22.91	19.60	32.08	30.23	32.11	31.64	30.83	29.92	21.52	18.41
W4_C1_F3	27.58	29.85	28.86	29.97	23.46	24.46	23.04	19.59	32.15	29.94	32.42	31.73	30.08	30.47	21.76	18.38
W4_C1_F3	27.65	29.98	28.78	29.83	23.39	24.39	22.93	19.62	31.98	30.12	32.36	30.82	30.38	30.64	21.61	18.48
W4_C2_F1	29.29	30.83	28.94	30.27	23.90	24.72	23.44	20.13	32.01	30.73	32.69	32.23	30.34	31.07	21.96	18.97
W4_C2_F1	28.32	30.39	28.95	30.27	23.96	24.64	23.35	20.11	31.86	30.98	32.96	31.54	30.63	31.06	21.99	18.95
W4_C2_F1	28.46	30.54	28.90	30.23	23.95	24.70	23.35	20.17	31.83	30.89	33.62	32.05	31.14	31.24	21.93	18.92
W4_C2_F2	27.85	29.50	28.50	29.76	23.54	24.62	22.98	19.71	32.82	30.40	32.71	30.57	30.16	30.64	21.63	18.51
W4_C2_F2	27.92	29.70	28.46	29.71	23.60	24.76	23.09	19.89	32.30	30.14	32.56	31.36	30.78	30.47	21.70	18.59
W4_C2_F2	28.00	29.36	28.31	29.73	23.60	24.58	23.03	19.77	31.36	30.13	32.24	31.38	30.35	30.38	21.64	18.54
W4_C2_F3	28.47	31.00	30.00	29.79	24.01	24.53	23.30	19.92	31.82	30.98	32.44	32.02	30.92	31.16	21.83	18.74
W4_C2_F3	28.38	30.88	30.03	30.02	24.00	24.44	23.19	19.83	32.38	30.00	33.26	32.50	30.77	31.01	21.83	18.70
W4_C2_F3	28.27	30.72	30.25	30.15	23.96	24.45	23.39	19.85	32.12	30.30	33.17	32.27	30.91	30.17	21.81	18.67
W4_V1_F1	28.98	30.29	29.06	30.50	24.05	25.18	23.63	20.24	32.31	30.87	32.96	31.83	30.89	30.58	22.13	19.07
W4_V1_F1	28.95	30.36	29.29	30.59	24.10	25.15	23.59	20.27	32.52	31.15	33.44	32.55	30.34	31.04	22.16	19.06
W4_V1_F1	28.48	30.33	29.10	30.40	24.07	25.12	23.62	20.24	32.95	30.26	32.96	31.57	30.14	30.31	22.10	19.03
W4_V1_F2	27.43	29.00	27.72	29.38	23.06	24.30	22.37	18.99	31.87	29.94	31.61	29.78	29.98	29.39	20.96	17.88
W4_V1_F2	27.51	28.95	27.65	29.13	23.12	24.29	22.34	19.09	32.03	29.44	31.44	30.31	30.29	30.24	20.96	17.99
W4_V1_F2	27.47	28.87	27.98	29.43	23.07	24.15	22.35	19.09	31.01	29.96	31.52	30.41	30.11	29.45	20.94	17.92

Sample	Study 3								Study 4							
	ccl20	***igha***	***ighb***	***irf7***	***cd74***	***saa5***	***etif3d***	***rpl32***	***cd4a***	***cd4b***	***il1b***	***tlr5a***	***tlr5b***	***tnfa***	***etif3d***	***rpl32***
W4_V1_F3	29.01	30.36	29.11	30.08	24.24	24.90	23.29	19.91	32.00	30.25	33.23	31.75	30.04	30.17	21.93	18.86
W4_V1_F3	28.85	29.80	29.11	30.13	24.13	24.79	23.27	19.99	31.57	30.05	33.40	31.96	30.56	30.57	21.90	18.81
W4_V1_F3	28.57	29.86	29.13	30.62	24.23	24.82	23.15	19.97	31.30	29.72	33.96	31.51	30.16	30.61	21.90	18.74
W4_V2_F1	28.67	30.18	28.78	30.36	24.37	24.80	23.12	19.86	31.66	29.98	32.02	32.09	30.31	30.58	21.72	18.61
W4_V2_F1	28.53	29.46	28.55	30.03	24.21	24.91	23.08	19.84	32.63	30.30	32.69	31.74	30.37	30.60	21.74	18.76
W4_V2_F1	28.58	29.42	28.49	29.93	24.12	24.90	23.21	19.93	31.62	30.05	32.21	32.55	30.39	30.57	21.61	18.69
W4_V2_F2	30.02	29.87	28.76	30.65	24.63	25.24	23.22	20.16	31.96	29.98	33.02	31.56	30.65	30.41	21.76	18.96
W4_V2_F2	28.93	29.72	29.23	30.44	24.76	25.21	23.26	20.13	31.57	30.00	33.38	32.31	30.06	30.66	21.77	18.82
W4_V2_F2	28.88	29.75	28.62	30.44	24.59	25.26	23.17	20.21	31.10	29.99	33.65	31.68	30.74	30.97	21.86	18.90
W4_V2_F3	28.10	29.79	29.02	29.62	No	24.62	23.09	19.77	31.55	29.84	31.54	30.43	30.36	30.08	21.72	18.53
W4_V2_F3	28.13	29.31	28.42	29.69	23.77	24.67	23.21	19.80	31.64	29.85	31.60	30.20	29.57	29.89	21.63	18.56
W4_V2_F3	28.07	29.55	28.53	29.62	23.65	24.56	23.15	19.83	30.92	29.90	32.05	30.25	29.93	29.90	21.66	18.51
linker_plate1	28.28	30.32	29.99	29.45	23.98	23.44	22.70	19.44	30.74	28.77	31.63	29.22	29.50	29.51	20.98	17.98
linker_plate1	28.26	30.15	30.75	29.42	24.05	23.40	22.76	19.48	30.76	29.07	31.64	29.11	29.48	29.17	21.00	18.07
linker_plate1	28.21	29.69	30.61	29.31	23.95	23.45	22.69	19.45	30.72	28.92	31.95	29.26	29.75	29.65	20.98	17.93
linker_plate2	28.42	29.99	30.08	29.11	23.98	23.42	22.73	19.52	30.94	29.08	31.77	29.46	29.51	29.08	21.09	18.04
linker_plate2	28.17	30.07	30.31	29.11	23.99	23.45	22.78	19.44	30.86	28.91	31.79	28.86	29.40	29.48	20.97	17.99
linker_plate2	28.31	29.57	30.09	29.32	24.08	23.42	22.76	19.47	31.51	29.29	31.09	29.69	29.79	29.30	21.01	17.94

Sample	Study 5							Study 6				
	ly6g6f	***cd8a***	***ighma***	***ighmc***	***infg***	***etif3d***	***rpl32***	***ighd***	***tlr7***	***rsad2***	***etif3d***	***rpl32***
W0_C1_F1	31.16	32.51	33.22	36.74	33.33	20.84	17.74	32.12	30.09	30.76	20.42	17.34
W0_C1_F1	32.42	32.79	33.18	35.40	33.65	20.85	17.71	32.07	29.99	30.44	20.44	17.40
W0_C1_F1	32.56	32.15	32.81	35.84	33.97	20.84	17.69	32.36	30.54	31.74	20.48	17.30
W0_C1_F2	32.84	32.37	33.28	36.25	33.80	20.84	17.75	32.44	30.03	30.58	20.58	17.46
W0_C1_F2	32.37	31.88	34.12	No	33.30	20.89	17.78	32.12	30.52	30.69	20.55	17.49
W0_C1_F2	31.91	32.38	33.43	36.04	34.04	20.98	17.77	31.80	30.29	31.24	20.60	17.44
W0_C1_F3	32.33	33.60	32.76	35.54	34.85	20.97	17.91	33.38	30.52	30.94	20.57	17.56
W0_C1_F3	31.68	33.95	33.64	No	34.16	20.90	17.87	33.02	30.41	30.70	20.63	17.62
W0_C1_F3	32.46	32.79	33.37	35.04	34.46	20.99	17.97	32.41	30.62	31.95	20.65	17.57
W2_C1_F1	31.59	30.75	32.42	33.96	31.50	20.83	17.81	29.83	30.89	31.71	20.52	17.45
W2_C1_F1	32.09	30.77	32.41	33.06	33.05	20.85	17.81	30.06	31.55	30.77	20.53	17.47
W2_C1_F1	31.66	30.67	32.31	33.52	31.43	20.81	17.84	29.96	31.26	31.77	20.47	17.40
W2_C1_F2	31.34	29.94	32.74	33.50	32.50	20.79	17.69	30.04	31.50	29.96	20.40	17.35
W2_C1_F2	31.87	29.83	32.38	33.10	31.16	20.68	17.72	29.98	31.61	30.14	20.34	17.37
W2_C1_F2	32.81	29.63	32.47	32.89	32.33	20.77	17.70	29.23	31.23	30.14	20.39	17.39
W2_C1_F3	30.85	29.64	32.29	33.64	31.72	20.62	17.62	29.98	30.94	32.55	20.25	17.28
W2_C1_F3	31.62	29.82	31.93	34.09	31.54	21.89	17.63	29.57	30.85	31.65	20.14	17.21
W2_C1_F3	30.60	29.97	32.01	32.35	31.68	20.76	17.61	29.71	30.48	31.56	20.25	17.29
W2_C2_F1	32.48	30.12	33.69	34.17	32.62	20.81	17.95	30.87	31.94	31.20	20.67	17.67
W2_C2_F1	31.96	30.15	33.14	34.07	31.99	20.83	17.99	31.64	31.70	31.32	20.58	17.72
W2_C2_F1	32.00	29.98	32.99	33.78	32.50	20.90	17.96	31.00	32.13	32.48	20.55	17.65
W2_C2_F2	32.07	29.85	32.37	33.24	31.50	20.60	17.71	30.02	31.81	29.90	20.18	17.35
W2_C2_F2	31.83	29.55	32.93	33.42	32.00	20.64	17.80	29.69	31.81	30.28	20.24	17.37

Sample	Study 5							Study 6				
	ly6g6f	*cd8a*	*ighma*	*ighmc*	*infg*	*etif3d*	*rpl32*	*ighd*	*tlr7*	*rsad2*	*etif3d*	*rpl32*
W2_C2_F2	31.42	29.69	32.55	33.51	31.92	20.62	17.71	29.83	32.02	30.35	20.27	17.30
W2_C2_F3	31.66	30.08	33.14	33.45	32.20	20.84	17.82	30.04	31.24	31.58	20.44	17.49
W2_C2_F3	31.77	29.95	32.39	34.67	31.56	20.84	17.87	30.06	30.91	31.83	20.43	17.47
W2_C2_F3	31.07	30.10	33.32	34.19	32.03	20.84	17.94	30.34	31.44	32.15	20.43	17.53
W2_V1_F1	32.72	29.52	32.18	31.08	30.40	21.31	18.05	30.46	31.74	31.30	20.97	17.81
W2_V1_F1	32.23	29.28	32.38	31.80	31.14	21.30	18.03	29.80	32.20	31.09	20.94	17.76
W2_V1_F1	31.98	29.60	32.34	31.40	31.14	21.27	18.21	30.14	32.81	31.45	20.91	17.83
W2_V1_F2	32.94	29.74	32.36	31.67	30.99	21.12	18.06	30.70	32.13	30.78	20.85	17.73
W2_V1_F2	32.98	29.57	32.69	31.79	30.80	21.29	18.10	30.70	31.91	30.56	20.94	17.81
W2_V1_F2	32.14	29.83	32.73	31.77	31.13	21.24	18.10	30.41	32.59	31.13	20.88	17.76
W2_V1_F3	32.92	30.34	32.64	33.91	31.31	21.38	18.25	30.81	32.76	30.96	20.99	17.93
W2_V1_F3	33.54	29.62	33.73	32.34	32.03	21.31	18.21	30.53	32.80	31.96	20.92	17.95
W2_V1_F3	33.49	29.96	32.88	33.33	31.70	21.34	18.26	30.93	32.58	31.37	20.98	17.94
W2_V2_F1	32.68	29.55	33.43	32.53	31.04	21.24	18.08	30.18	32.19	31.87	20.84	17.70
W2_V2_F1	32.57	29.59	32.63	32.50	31.57	21.27	18.02	30.31	32.14	31.79	20.89	17.76
W2_V2_F1	31.55	29.09	32.37	32.46	31.46	21.25	17.98	30.14	32.51	31.31	20.89	17.66
W2_V2_F2	33.24	30.03	34.00	34.90	31.13	21.19	18.24	31.33	32.72	31.63	20.80	17.83
W2_V2_F2	31.84	29.79	33.59	34.08	32.16	21.29	18.20	31.20	32.59	31.23	20.87	17.81
W2_V2_F2	33.67	30.01	34.11	34.05	33.23	21.20	18.22	31.41	32.65	31.43	20.96	17.93
W2_V2_F3	33.19	29.99	33.01	32.49	31.17	21.29	18.19	31.34	32.96	32.37	20.94	17.99
W2_V2_F3	33.10	30.08	33.22	33.50	31.47	21.23	18.23	30.50	No	32.09	20.82	17.85
W2_V2_F3	32.49	29.77	33.02	33.48	32.19	21.20	18.28	31.27	32.37	31.57	20.99	18.57
W4_C1_F1	34.70	29.55	33.78	33.23	32.68	22.02	18.99	31.18	33.00	32.45	21.79	18.83

Sample	Study 5							Study 6				
	ly6g6f	*cd8a*	*ighma*	*ighmc*	*infg*	*etif3d*	*rpl32*	*ighd*	*tlr7*	*rsad2*	*etif3d*	*rpl32*
W4_C1_F1	32.67	29.59	33.09	33.28	31.75	22.09	19.13	31.38	34.65	32.47	21.81	18.79
W4_C1_F1	36.24	29.27	33.85	32.86	31.88	22.08	18.98	31.03	34.25	32.81	21.76	18.71
W4_C1_F2	33.61	28.92	32.99	31.72	30.52	21.76	18.55	30.39	33.22	33.15	21.42	18.10
W4_C1_F2	32.65	29.66	32.65	32.03	31.16	21.86	18.54	30.42	33.25	31.28	21.42	18.38
W4_C1_F2	33.66	29.17	32.49	31.73	30.75	21.85	18.68	31.01	34.01	32.32	21.70	18.49
W4_C1_F3	33.47	29.40	32.82	32.12	31.40	21.77	18.49	31.07	33.16	31.79	21.49	18.23
W4_C1_F3	36.13	29.17	33.14	32.25	30.82	21.80	18.66	31.83	33.17	31.68	21.72	18.34
W4_C1_F3	33.18	29.06	33.05	33.18	30.99	21.76	18.57	31.16	34.95	30.88	21.38	18.21
W4_C2_F1	33.81	30.24	33.65	32.86	32.83	22.11	19.10	31.77	34.76	32.70	21.70	18.84
W4_C2_F1	34.96	30.04	33.96	32.75	31.49	22.15	19.13	31.94	36.29	31.77	21.78	18.76
W4_C2_F1	33.20	29.96	33.98	33.08	31.64	22.06	19.05	32.45	No	31.71	21.75	18.77
W4_C2_F2	34.03	29.62	32.76	32.22	31.46	21.70	18.72	30.76	33.75	32.62	21.45	18.33
W4_C2_F2	32.98	29.46	32.91	31.63	31.95	21.71	18.73	31.11	34.02	31.28	21.39	18.41
W4_C2_F2	32.78	29.40	32.79	32.31	31.94	21.91	18.65	31.09	33.63	32.10	21.37	18.40
W4_C2_F3	33.89	29.51	33.91	32.76	31.68	22.13	18.86	31.35	33.42	34.99	21.74	18.61
W4_C2_F3	37.29	28.92	33.90	33.20	32.39	22.11	18.84	31.40	33.90	31.50	21.86	18.60
W4_C2_F3	34.03	29.48	33.52	34.26	31.00	22.06	18.86	31.25	34.55	33.38	21.66	18.59
W4_V1_F1	32.71	30.19	33.43	33.56	31.60	22.43	19.17	32.37	34.66	33.59	21.92	18.95
W4_V1_F1	34.22	29.64	33.29	32.12	31.87	22.38	19.23	32.21	35.12	33.23	22.22	18.98
W4_V1_F1	33.11	30.20	33.16	32.98	31.79	22.27	19.19	31.67	34.99	34.17	21.93	18.98
W4_V1_F2	32.91	28.94	32.44	31.41	30.42	20.98	17.99	30.73	32.64	31.13	20.56	17.70
W4_V1_F2	32.21	29.10	32.96	31.55	30.31	21.01	18.11	30.83	33.54	32.92	20.68	17.65
W4_V1_F2	32.54	29.17	32.80	31.11	30.24	20.63	18.02	30.64	33.74	32.94	20.80	17.80

Sample	Study 5							Study 6				
	ly6g6f	*cd8a*	*ighma*	*ighmc*	*infg*	*etif3d*	*rpl32*	*ighd*	*tlr7*	*rsad2*	*etif3d*	*rpl32*
W4_V1_F3	33.44	29.51	32.77	32.70	31.56	21.88	18.96	31.22	34.11	32.09	21.64	18.70
W4_V1_F3	34.49	29.62	33.43	32.54	31.55	21.98	18.96	31.74	34.51	33.14	21.64	18.69
W4_V1_F3	35.47	29.21	33.11	33.09	31.43	21.97	18.90	31.69	34.56	32.66	21.62	18.67
W4_V2_F1	33.40	29.21	33.47	32.12	31.97	21.86	18.83	30.65	33.60	30.11	21.48	18.52
W4_V2_F1	33.53	29.31	33.41	31.83	31.32	21.87	19.01	31.26	33.40	29.99	21.49	18.49
W4_V2_F1	34.47	29.15	32.94	32.22	31.71	21.87	18.87	30.87	34.65	29.75	21.48	18.50
W4_V2_F2	35.69	29.44	33.39	32.81	31.58	21.88	19.03	31.07	34.15	32.67	21.53	18.70
W4_V2_F2	34.36	29.36	33.69	32.04	32.29	21.97	19.03	31.43	34.30	31.69	28.35	18.78
W4_V2_F2	33.76	29.32	32.80	32.44	32.12	21.96	19.16	31.31	33.42	34.84	21.55	18.78
W4_V2_F3	32.77	29.28	33.17	31.71	30.81	21.88	18.71	30.65	34.57	31.15	21.41	18.36
W4_V2_F3	33.32	28.76	33.61	31.65	30.80	21.78	18.78	30.39	33.33	30.80	21.43	18.35
W4_V2_F3	33.00	29.22	32.82	31.11	30.70	21.82	18.70	30.38	33.54	31.18	21.42	18.40
linker_plate1	31.85	29.43	32.54	31.33	31.00	21.25	18.13	30.13	31.97	31.35	20.88	17.83
linker_plate1	32.21	29.25	32.85	31.41	30.89	21.20	18.17	30.20	32.77	30.89	20.90	17.85
linker_plate1	33.00	29.72	32.75	31.77	30.84	21.18	18.08	30.42	32.89	30.74	20.84	17.74
linker_plate2	32.33	29.10	32.33	31.34	31.32	21.16	18.15	30.32	32.93	30.23	20.77	17.75
linker_plate2	31.69	29.21	32.67	31.07	30.75	21.19	18.10	30.43	32.93	30.24	20.81	17.70
linker_plate2	32.62	29.24	32.88	31.94	31.00	21.21	18.08	30.28	32.65	30.86	20.90	17.78

Dropped tech reps: out of range

*Each value represents the mean of technical replicates (n=3).

Appendix II. Transcript expression levels of immune-related genes in lumpfish (*Cyclopterus lumpus*) larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii calculated using the $2^{(-\Delta\Delta Ct)}$ method and $\log_2$ converted.

Sample	*ccl19*	*cox2*	*hamp*	*il8a*	*il8b*	*il10*	*lgp2*	*mxa*	*mxb*	*mxc*	*stat1*	*tlr3*	*ccl20*	*igha*	*ighb*	*irf7*
W0_C1_F1	0.14	-0.09	0.26	0.16	0.05	0.24	-0.06	-0.21	-0.38	-0.32	0.08	0.09	0.21	0.01	0.32	0.08
W0_C1_F2	0.08	0.16	-0.13	-0.11	-0.09	-0.05	0.15	0.08	0.21	0.17	-0.03	0.21	-0.22	-0.36	-0.40	0.11
W0_C1_F3	-0.22	-0.07	-0.13	-0.05	0.04	-0.19	-0.09	0.13	0.17	0.15	-0.05	-0.30	0.01	0.34	0.07	-0.19
W0_Ctrl_Avg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
W2_C1_F1	-0.15	0.11	0.30	0.05	0.56	0.29	0.05	-0.05	-0.25	-0.06	0.04	0.18	0.02	0.11	0.28	0.07
W2_C1_F2	-0.08	0.04	0.24	0.04	-0.14	0.01	0.03	0.09	0.56	0.30	0.06	0.23	0.02	0.50	0.14	0.05
W2_C1_F3	0.49	0.24	0.26	0.27	0.33	0.34	0.06	0.05	0.04	0.09	0.00	0.44	0.18	0.94	0.23	0.41
W2_C2_F1	0.00	-0.17	-0.46	-0.37	-0.21	0.27	0.02	0.12	-0.20	-0.23	0.15	-0.29	-0.23	-0.78	-0.67	-0.22
W2_C2_F2	-0.06	-0.05	-0.13	-0.13	-0.31	-0.46	0.10	0.00	0.03	0.36	-0.04	-0.18	0.14	-0.27	-0.10	-0.06
W2_C2_F3	-0.20	**-0.17**	-0.22	0.15	-0.22	-0.44	-0.25	-0.22	-0.17	-0.47	-0.21	-0.39	-0.13	-0.52	0.12	-0.25
W2_Ctrl_Avg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
W2_V1_F1	1.52	0.28	0.10	0.80	0.86	1.02	0.89	0.35	0.68	0.59	0.70	0.41	0.86	2.08	1.70	0.51
W2_V1_F2	1.33	0.26	-0.15	0.44	0.91	1.31	0.70	0.14	0.36	0.32	0.44	0.65	0.64	1.54	1.16	0.36
W2_V1_F3	0.85	0.02	-0.58	-0.03	0.95	0.34	0.34	-0.13	0.20	-0.28	0.13	0.41	0.57	0.86	0.59	0.27
W2_V2_F1	0.84	0.34	0.09	0.29	0.27	1.02	0.52	-0.04	0.28	0.35	0.10	0.47	0.30	1.81	0.95	0.32
W2_V2_F2	0.40	-0.11	-0.38	-0.71	0.50	0.11	0.20	-0.20	0.27	-0.33	0.11	-0.19	0.02	0.23	0.26	-0.52
W2_V2_F3	0.66	**0.05**	-0.19	-0.27	0.03	0.33	-0.09	-0.38	-0.23	-0.42	-0.29	-0.38	0.44	0.97	0.55	-0.08
W2_VA_Avg	**0.93**	**0.14**	**-0.19**	**0.09**	**0.59**	**0.69**	**0.43**	**-0.04**	**0.26**	**0.04**	**0.20**	**0.23**	**0.47**	**1.25**	**0.87**	**0.14**
W4_C1_F1	-0.38	-0.02	-0.01	-0.10	-0.60	0.19	-0.21	-0.32	-0.08	-0.29	0.06	0.08	0.13	-0.28	-0.39	-0.06
W4_C1_F2	0.46	0.45	-0.12	-0.29	0.09	0.78	0.15	0.05	0.20	0.00	-0.06	0.16	0.20	0.66	0.15	-0.11
W4_C1_F3	-0.45	0.41	0.20	-0.08	-0.05	0.69	-0.09	0.08	-0.11	0.03	-0.25	-0.31	0.12	0.08	0.06	-0.18

Sample	*ccl19*	*cox2*	*hamp*	*il8a*	*il8b*	*il10*	*lgp2*	*mxa*	*mxb*	*mxc*	*stat1*	*tlr3*	*ccl20*	*igha*	*ighb*	*irf7*
W4_C2_F1	0.22	-0.33	0.17	0.64	0.14	-0.94	-0.02	0.07	0.05	0.30	0.01	0.10	-0.14	-0.24	0.45	0.03
W4_C2_F2	-0.10	-0.22	0.09	-0.05	-0.07	-0.20	0.10	0.32	0.30	0.26	0.06	0.16	-0.02	0.48	0.61	0.21
W4_C2_F3	0.25	**-0.29**	-0.33	-0.12	0.49	-0.52	0.07	-0.20	-0.36	-0.30	0.18	-0.19	-0.30	-0.70	-0.89	0.12
W4_Ctrl_Avg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
W4_V1_F1	0.63	-0.10	0.33	0.02	-0.46	1.22	-0.05	-0.06	-0.07	0.05	0.03	0.01	-0.54	0.20	0.41	-0.04
W4_V1_F2	-0.04	-0.20	0.21	-0.16	0.24	1.10	-0.29	-0.32	-0.33	-0.24	-0.38	-0.14	-0.27	0.36	0.54	-0.08
W4_V1_F3	0.30	-0.30	-0.05	-0.12	0.03	0.39	-0.40	-0.38	-0.36	-0.47	-0.15	-0.27	-0.72	0.18	0.10	0.02
W4_V2_F1	0.54	-0.02	0.40	0.54	0.19	1.17	0.03	0.48	1.05	1.32	-0.02	-0.03	-0.59	0.65	0.52	-0.07
W4_V2_F2	-0.37	-0.53	0.14	0.28	-0.34	0.43	-0.39	-0.47	-0.27	-0.13	-0.04	-0.30	-0.72	0.50	0.63	-0.29
W4_V2_F3	0.41	**0.34**	0.24	0.60	0.61	1.36	-0.01	-0.02	-0.14	0.20	0.16	0.34	-0.13	0.51	0.62	0.36
W4_VA_Avg	**0.24**	**-0.14**	**0.21**	**0.19**	**0.05**	**0.94**	**-0.18**	**-0.13**	**-0.02**	**0.12**	**-0.07**	**-0.07**	**-0.49**	**0.40**	**0.47**	**-0.02**

Sample	*cd74*	*saa5*	*cd4a*	*cd4b*	*il1b*	*tlr5a*	*tlr5b*	*tnfa*	*ly6g6f*	*cd8a*	*ighma*	*ighmc*	*infg*	*ighd*	*tlr7*	*rsad2*
W0_C1_F1	0.07	0.09	-0.05	0.45	-0.44	0.15	0.62	-0.03	-0.14	0.27	0.09	0.00	0.23	0.12	0.02	-0.02
W0_C1_F2	0.03	0.19	-0.07	-0.31	1.09	-0.09	-0.45	0.28	0.03	0.60	-0.14	-0.47	0.22	0.30	0.07	0.06
W0_C1_F3	-0.10	-0.28	0.12	-0.14	-0.65	-0.05	-0.17	-0.25	0.11	-0.87	0.06	0.48	-0.45	-0.43	-0.09	-0.04
W0_Ctrl_Avg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
W2_C1_F1	-0.03	-0.12	-0.36	-0.04	-0.17	-0.52	0.24	-0.14	-0.10	-0.65	0.35	0.07	0.53	0.22	0.23	-0.56
W2_C1_F2	0.09	-0.08	0.26	0.16	0.06	-0.25	-0.08	0.08	-0.04	0.17	0.10	0.31	-0.53	0.06	-0.08	1.00
W2_C1_F3	0.10	-0.59	0.15	0.25	0.41	0.19	0.48	0.43	0.78	0.10	0.49	0.42	0.17	0.18	0.48	-0.66
W2_C2_F1	-0.08	1.62	0.15	-0.05	-0.14	1.15	-0.26	-0.40	-0.39	0.08	-0.45	-0.34	-0.30	-0.60	-0.29	0.08
W2_C2_F2	0.05	-0.44	-0.33	0.07	-0.19	-0.26	-0.12	-0.24	-0.25	0.23	-0.03	0.04	0.03	0.13	-0.60	0.81

Sample	*cd74*	*saa5*	*cd4a*	*cd4b*	*il1b*	*tlr5a*	*tlr5b*	*tnfa*	*ly6g6f*	*cd8a*	*ighma*	*ighmc*	*infg*	*ighd*	*tlr7*	*rsad2*
W2_C2_F3	-0.13	-0.39	0.14	-0.39	0.04	-0.31	-0.26	0.28	-0.01	0.07	-0.46	-0.49	0.09	0.01	0.26	-0.68
W2_Ctrl_Avg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
W2_V1_F1	1.00	2.83	0.32	0.16	0.94	2.41	0.82	0.91	-0.27	0.98	0.81	2.53	1.22	0.43	-0.39	0.30
W2_V1_F2	0.73	2.21	-0.23	-0.01	0.72	1.71	0.10	0.68	-0.96	0.69	0.47	2.17	1.34	-0.08	-0.39	0.71
W2_V1_F3	0.42	-0.34	-0.20	-0.41	0.36	-0.26	-0.63	0.48	-1.18	0.57	0.44	0.43	0.77	-0.11	-0.77	0.23
W2_V2_F1	0.60	-0.52	-0.25	0.10	-0.47	-0.14	-0.02	0.19	-0.64	0.98	0.55	1.40	0.95	0.27	-0.50	-0.16
W2_V2_F2	0.31	0.37	0.13	-0.68	-0.51	-1.04	-1.13	-0.52	-1.38	0.53	-0.77	-0.08	0.21	-0.75	-0.79	0.14
W2_V2_F3	0.00	-0.27	0.02	-0.20	-0.18	-0.91	-0.78	-0.35	-1.06	0.54	0.06	0.50	1.08	-0.69	-0.75	-0.38
W2_VA_Avg	**0.51**	**0.71**	**-0.04**	**-0.17**	**0.14**	**0.30**	**-0.27**	**0.23**	**-0.91**	**0.72**	**0.26**	**1.16**	**0.93**	**-0.16**	**-0.60**	**0.14**
W4_C1_F1	-0.04	-0.25	0.24	0.15	-0.13	0.30	0.15	0.04	-0.72	0.21	-0.31	-0.40	-0.18	0.16	-0.22	-0.05
W4_C1_F2	0.08	0.31	0.35	0.51	0.55	0.23	-0.02	0.35	-0.16	0.08	0.45	0.55	0.48	0.59	0.64	-0.20
W4_C1_F3	0.06	-0.08	-0.25	-0.11	0.13	-0.19	-0.04	-0.02	0.12	0.09	0.13	0.16	0.19	-0.14	0.68	0.57
W4_C2_F1	0.04	0.10	0.36	-0.46	0.02	-0.02	0.12	-0.17	0.36	-0.35	-0.31	-0.12	0.12	-0.51	-1.30	0.65
W4_C2_F2	0.05	-0.22	-0.66	-0.17	-0.01	0.20	0.03	0.11	0.63	-0.13	0.38	0.14	-0.46	-0.02	0.04	0.02
W4_C2_F3	-0.19	0.13	-0.04	0.07	-0.56	-0.53	-0.24	-0.31	-0.22	0.11	-0.34	-0.33	-0.13	-0.08	0.16	-0.99
W4_Ctrl_Avg	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
W4_V1_F1	0.08	-0.19	-0.20	-0.21	-0.13	-0.17	0.71	0.45	1.13	-0.11	0.44	0.07	0.11	-0.71	-0.48	-1.04
W4_V1_F2	-0.15	-0.51	-0.70	-0.38	0.31	-0.03	-0.32	0.53	0.17	-0.49	-0.32	0.27	0.21	-0.46	-0.49	-1.60
W4_V1_F3	-0.38	-0.21	0.54	0.31	-0.78	-0.24	0.47	0.41	-0.76	0.12	0.30	-0.16	0.01	-0.31	-0.28	-0.34
W4_V2_F1	-0.50	-0.34	0.35	0.03	0.27	0.15	0.20	0.11	0.19	0.29	0.07	0.51	-0.20	0.15	0.45	2.18
W4_V2_F2	-0.75	-0.52	0.61	0.31	-0.61	-0.15	0.01	0.17	-0.96	0.26	-0.08	0.25	-0.62	-0.04	-0.13	0.09
W4_V2_F3	-0.02	-0.11	0.31	0.19	0.77	0.48	0.51	0.65	0.51	0.15	0.03	0.77	0.59	0.50	0.41	0.98
W4_VA_Avg	**-0.29**	**-0.31**	**0.15**	**0.04**	**-0.03**	**0.01**	**0.26**	**0.39**	**0.05**	**0.04**	**0.08**	**0.28**	**0.02**	**-0.15**	**-0.09**	**0.04**

Appendix III. RQ values of transcript expression levels of immune-related genes in lumpfish (*Cyclopterus lumpus*) larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii.

Sample	*ccl19*	*cox2*	*hamp*	*il8a*	*il8b*	*il10*	*lgp2*	*mxa*	*mxb*	*mxc*	*stat1*	*tlr3*	*ccl20*	*igha*	*ighb*	*irf7*
W0_C1_F1	1.28	3.88	1.35	1.87	1.34	3.61	1.06	1.02	1.17	1.22	1.09	1.30	1.29	1.28	1.60	1.17
W0_C1_F2	1.23	4.56	1.04	1.54	1.21	2.95	1.21	1.22	1.71	1.66	1.02	1.39	1.00	1.00	1.00	1.19
W0_C1_F3	1.00	3.89	1.03	1.60	1.31	2.69	1.05	1.26	1.67	1.63	1.00	1.00	1.15	1.57	1.35	1.00
W0_Ctrl_Avg	**1.17**	**4.11**	**1.14**	**1.67**	**1.29**	**3.08**	**1.11**	**1.17**	**1.52**	**1.50**	**1.04**	**1.23**	**1.14**	**1.28**	**1.32**	**1.12**
W2_C1_F1	2.18	1.78	1.84	1.76	2.91	1.82	1.20	1.23	1.00	1.30	1.44	1.76	2.02	6.94	6.04	1.88
W2_C1_F2	2.31	1.71	1.78	1.77	1.82	1.52	1.18	1.34	1.73	1.65	1.46	1.82	2.03	8.89	5.50	1.86
W2_C1_F3	3.38	1.97	1.81	2.08	2.53	1.91	1.20	1.31	1.22	1.43	1.41	2.08	2.23	11.89	5.84	2.29
W2_C2_F1	2.39	1.44	1.11	1.29	1.70	1.78	1.18	1.36	1.02	1.15	1.53	1.28	1.76	3.85	3.23	1.58
W2_C2_F2	2.32	1.60	1.39	1.55	1.61	1.10	1.23	1.26	1.21	1.70	1.38	1.39	2.18	5.31	4.61	1.74
W2_C2_F3	2.11	1.46	1.31	1.88	1.70	1.11	1.00	1.11	1.06	1.00	1.24	1.22	1.85	4.58	5.42	1.55
W2_Ctrl_Avg	**2.45**	**1.66**	**1.54**	**1.72**	**2.05**	**1.54**	**1.17**	**1.27**	**1.20**	**1.37**	**1.41**	**1.59**	**2.01**	**6.91**	**5.11**	**1.82**
W2_V1_F1	6.47	1.94	1.59	2.86	3.48	2.91	1.99	1.57	1.83	1.95	2.17	2.02	3.36	24.31	14.82	2.44
W2_V1_F2	5.66	1.88	1.32	2.20	3.55	3.49	1.77	1.37	1.46	1.62	1.83	2.32	2.95	17.16	10.46	2.24
W2_V1_F3	4.09	1.59	1.00	1.58	3.63	1.81	1.43	1.16	1.31	1.10	1.52	1.99	2.83	10.97	7.18	2.12
W2_V2_F1	4.12	2.02	1.56	2.00	2.32	2.89	1.59	1.23	1.39	1.66	1.49	2.08	2.41	20.31	9.13	2.19
W2_V2_F2	3.07	1.47	1.15	1.00	2.71	1.57	1.32	1.11	1.38	1.07	1.50	1.36	2.05	7.29	5.82	1.33
W2_V2_F3	3.57	1.60	1.28	1.32	1.92	1.78	1.11	1.00	1.00	1.01	1.17	1.21	2.63	11.74	6.97	1.72
W2_VA_Avg	**4.50**	**1.75**	**1.32**	**1.83**	**2.94**	**2.41**	**1.54**	**1.24**	**1.40**	**1.40**	**1.61**	**1.83**	**2.70**	**15.30**	**9.06**	**2.01**
W4_C1_F1	3.84	1.43	1.70	1.14	1.00	2.12	1.40	1.41	1.48	1.21	1.77	1.33	3.64	15.72	19.16	1.87
W4_C1_F2	6.95	2.08	1.63	1.05	1.68	3.27	1.73	1.77	1.82	1.49	1.65	1.42	3.78	29.39	27.66	1.82
W4_C1_F3	3.76	1.99	2.01	1.21	1.51	3.06	1.50	1.81	1.48	1.52	1.47	1.05	3.59	20.17	26.14	1.74

Sample	*ccl19*	*cox2*	*hamp*	*il8a*	*il8b*	*il10*	*lgp2*	*mxa*	*mxb*	*mxc*	*stat1*	*tlr3*	*ccl20*	*igha*	*ighb*	*irf7*
W4_C2_F1	5.75	1.16	1.93	1.93	1.67	1.00	1.56	1.78	1.61	1.77	1.71	1.34	3.11	16.07	32.94	1.98
W4_C2_F2	4.76	1.29	1.86	1.22	1.49	1.68	1.68	2.10	1.92	1.75	1.77	1.41	3.32	25.98	37.03	2.20
W4_C2_F3	5.94	1.21	1.40	1.15	2.14	1.34	1.65	1.52	1.24	1.21	1.90	1.12	2.83	12.03	13.97	2.09
W4_Ctrl_Avg	**5.17**	**1.53**	**1.76**	**1.28**	**1.58**	**2.08**	**1.59**	**1.73**	**1.59**	**1.49**	**1.71**	**1.28**	**3.38**	**19.89**	**26.15**	**1.95**
W4_V1_F1	7.43	1.33	2.11	1.22	1.09	4.18	1.54	1.65	1.48	1.50	1.74	1.26	2.47	21.18	31.83	1.91
W4_V1_F2	5.15	1.39	2.10	1.22	1.94	4.21	1.32	1.42	1.32	1.30	1.36	1.19	2.83	24.71	36.46	1.84
W4_V1_F3	6.08	1.19	1.66	1.13	1.55	2.44	1.25	1.35	1.23	1.08	1.55	1.06	2.21	21.25	26.42	1.97
W4_V2_F1	7.18	1.46	2.26	1.83	1.75	4.15	1.61	2.29	3.13	3.44	1.68	1.24	2.37	28.98	34.82	1.86
W4_V2_F2	3.84	1.00	1.88	1.47	1.19	2.48	1.25	1.28	1.30	1.34	1.65	1.04	2.21	25.95	36.90	1.63
W4_V2_F3	6.60	1.87	2.05	1.90	2.34	4.72	1.57	1.69	1.45	1.68	1.89	1.58	3.12	26.53	37.27	2.41
W4_VA_Avg	**6.05**	**1.38**	**2.01**	**1.46**	**1.64**	**3.70**	**1.42**	**1.61**	**1.65**	**1.72**	**1.64**	**1.23**	**2.54**	**24.77**	**33.95**	**1.94**

Sample	*cd74*	*saa5*	*cd4a*	*cd4b*	*il1b*	*tlr5a*	*tlr5b*	*tnfa*	*ly6g6f*	*cd8a*	*ighma*	*ighmc*	*infg*	*ighd*	*tlr7*	*rsad2*
W0_C1_F1	1.13	1.30	1.51	1.87	1.17	3.30	1.96	1.48	1.73	2.08	1.30	1.35	1.57	1.43	13.44	4.11
W0_C1_F2	1.10	1.38	1.49	1.13	3.32	2.82	1.00	1.80	1.92	2.56	1.12	1.00	1.56	1.59	13.79	4.32
W0_C1_F3	1.00	1.00	1.67	1.25	1.00	2.86	1.19	1.27	2.01	1.00	1.27	1.82	1.00	1.00	12.44	4.00
W0_Ctrl_Avg	**1.08**	**1.22**	**1.56**	**1.41**	**1.83**	**2.99**	**1.38**	**1.52**	**1.89**	**1.88**	**1.23**	**1.39**	**1.38**	**1.34**	**13.22**	**4.15**
W2_C1_F1	3.29	1.75	1.48	1.98	1.64	1.84	2.46	1.48	2.78	6.58	2.08	5.21	6.63	6.14	7.25	1.97
W2_C1_F2	3.54	1.78	2.18	2.28	1.93	2.21	2.01	1.70	2.91	11.19	1.78	6.09	3.36	5.56	5.96	5.78
W2_C1_F3	3.59	1.27	2.04	2.43	2.46	2.98	2.87	2.14	4.89	10.70	2.29	3.77	5.31	6.02	8.55	1.88
W2_C2_F1	3.10	5.50	2.03	1.96	1.66	5.59	1.79	1.24	2.31	10.46	1.23	4.01	3.87	3.63	5.13	3.02
W2_C2_F2	3.34	1.36	1.51	2.14	1.94	2.19	1.96	1.39	2.55	11.65	1.64	5.13	4.84	5.84	4.25	5.12

Sample	*cd74*	*saa5*	*cd4a*	*cd4b*	*il1b*	*tlr5a*	*tlr5b*	*tnfa*	*ly6g6f*	*cd8a*	*ighma*	*ighmc*	*infg*	*ighd*	*tlr7*	*rsad2*
W2_C2_F3	3.02	1.43	2.02	1.57	1.90	2.11	1.79	1.94	2.95	10.42	1.23	3.66	4.99	5.38	7.39	1.81
W2_Ctrl_Avg	**3.31**	**2.18**	**1.88**	**2.06**	**1.92**	**2.82**	**2.15**	**1.65**	**3.06**	**10.17**	**1.71**	**4.65**	**4.83**	**5.43**	**6.42**	**3.26**
W2_V1_F1	6.39	12.31	2.25	2.21	3.40	12.76	3.53	2.87	2.49	18.54	2.76	24.32	10.21	6.94	4.79	3.44
W2_V1_F2	5.30	8.08	1.60	1.98	2.94	8.02	2.23	2.47	1.61	15.38	2.22	19.41	11.06	5.04	4.80	4.59
W2_V1_F3	4.26	1.44	1.64	1.50	2.26	2.11	1.40	2.16	1.39	14.17	2.16	6.47	7.64	4.92	3.73	3.25
W2_V2_F1	4.84	1.29	1.58	2.13	1.30	2.30	2.06	1.79	1.97	18.54	2.34	11.97	8.60	6.31	4.48	2.52
W2_V2_F2	3.94	2.32	2.00	1.26	1.25	1.25	1.02	1.14	1.23	13.87	1.00	4.70	5.32	3.28	3.68	3.09
W2_V2_F3	3.18	1.51	1.87	1.73	1.57	1.36	1.27	1.27	1.50	13.95	1.70	6.77	9.32	3.40	3.77	2.15
W2_VA_Avg	**4.65**	**4.49**	**1.82**	**1.80**	**2.12**	**4.64**	**1.92**	**1.95**	**1.70**	**15.74**	**2.03**	**12.27**	**8.69**	**4.98**	**4.21**	**3.17**
W4_C1_F1	7.43	1.56	1.77	1.50	2.04	1.72	2.24	1.25	1.02	30.96	1.73	13.95	11.07	6.14	2.01	2.45
W4_C1_F2	8.31	2.34	1.90	1.96	3.35	1.69	2.04	1.55	1.47	28.98	2.88	25.71	17.20	8.18	3.55	2.46
W4_C1_F3	8.21	1.81	1.32	1.29	2.50	1.28	2.01	1.22	1.75	29.09	2.34	20.08	14.29	5.13	3.66	3.46
W4_C2_F1	7.85	1.98	1.90	1.00	2.25	1.39	2.19	1.09	2.02	21.61	1.74	16.61	13.41	4.01	1.00	4.31
W4_C2_F2	8.09	1.63	1.02	1.24	2.27	1.64	2.10	1.32	2.41	25.28	2.74	19.84	9.36	5.54	2.42	2.87
W4_C2_F3	6.81	2.05	1.49	1.44	1.54	1.00	1.76	1.00	1.41	29.21	1.71	14.64	11.50	5.29	2.59	1.41
W4_Ctrl_Avg	**7.78**	**1.90**	**1.57**	**1.40**	**2.32**	**1.45**	**2.05**	**1.24**	**1.68**	**27.52**	**2.19**	**18.47**	**12.80**	**5.72**	**2.54**	**2.83**
W4_V1_F1	7.96	1.61	1.35	1.17	2.01	1.78	3.19	1.62	3.28	25.20	2.80	18.68	13.25	3.51	1.69	1.32
W4_V1_F2	7.40	1.40	1.00	1.11	2.96	2.18	1.71	1.76	1.84	20.57	1.79	21.86	14.90	4.28	1.76	1.00
W4_V1_F3	5.95	1.62	2.13	1.67	1.32	1.50	2.74	1.59	1.00	29.43	2.57	16.25	12.59	4.56	1.94	2.19
W4_V2_F1	5.52	1.50	1.90	1.41	2.73	1.04	2.32	1.31	1.82	32.85	2.24	24.80	11.04	6.14	3.13	12.51
W4_V2_F2	4.62	1.30	2.21	1.67	1.48	1.61	2.05	1.36	1.34	32.03	2.02	20.99	8.34	5.40	2.14	2.96
W4_V2_F3	7.70	1.75	1.85	1.57	3.84	3.39	2.84	1.87	2.24	30.25	2.19	29.38	18.47	7.72	3.07	5.56
W4_VA_Avg	**6.53**	**1.53**	**1.74**	**1.43**	**2.39**	**1.92**	**2.48**	**1.59**	**1.92**	**28.39**	**2.27**	**21.99**	**13.10**	**5.27**	**2.29**	**4.26**

*Each value represents the mean of technical replicates (n=3).