Innate antiviral-like immune responses differ among divergent European and North American farmed and wild Atlantic salmon (*Salmo salar*) and their hybrids

by

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ABSTRACT

This thesis involved a study of the effect of North American (NA) farmed, European origin (EO) farmed, wild, and related hybrid cross types on the innate antiviral-like immune response in Atlantic salmon (*Salmo salar*). Since the 1970s, millions of farmed salmon have escaped causing concern about how interbreeding with wild salmon may affect wild populations. Farmed and wild salmon differ genetically, yet the immune responses of farmed-wild hybrids are not well understood. In Newfoundland, permission has been granted to start farming an EO strain in addition to the current NA strain from the Saint John River. As these strains differ genetically and geographically, there is concern about potential differences in immune responses. Hence, I used real-time quantitative polymerase chain reaction (qPCR) with head kidney templates to measure expression of seventeen antiviral and other immune-relevant biomarker genes (i.e., *dhx58, gig1a, helz2, ifna, ifng, irf1a, irf7b, isg15a, mxb, pgds, rsad2a, rsad2b, stat1a, stat1c, tlr3, tlr7, and 5loxb*) to assess the innate antiviral-like immune responses [i.e. to intraperitoneal (IP) injection with polyriboinosinic polyribocytidylic (pIC)] of six different Atlantic salmon cross types including pure crosses (i.e. NA, EO, and local Northeast Placentia River Wild) and three related hybrid crosses. Cross type abbreviations are as follows: pure Wild; pure European farmed (Farm.EO); pure North American farmed (Farm.NA); wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$); Farm.EO female crossed with wild male (Hyb.EO $_{F♀}$); and Farm.NA female crossed with wild male (Hyb.NA $_{F♀}$). At a basal transcript expression level, only one gene (*pgds*) showed significant cross type differences, where Hyb.NA $_{F♀}$ had significantly higher expression than the Wild and Hyb.EO $_{F♀}$. After pIC injection, fifteen of the seventeen target genes (i.e. all except *pgds* and *5loxb*) were significantly upregulated. In addition, there were significant cross type differences in transcript expression of *ifna*, *isg15a*,

rsad2a, and *rsad2b* 24 hours post-injection, but only for pIC-injected fish and not the control fish (i.e., sterile phosphate buffered saline injected). The transcript expression of *ifna* was significantly higher in Farm.EO compared to Wild, Hyb.EO $_{F♀}$, and Hyb.NA$_{F♀}$, and also higher in Farm.NA than Wild. The transcript expression of both *isg15a* and *rsad2a* was significantly higher in Farm.EO and Hyb.EO $_{W♀}$ than Wild and Hyb.NA $_{F♀}$. Lastly, the transcript expression of *rsad2b* was significantly higher in Hyb.EO $_{W♀}$ than Wild. This experiment identified important differences in innate antiviral-like response among wild, farmed and related hybrid Atlantic salmon. The results from this study support the hypothesis that interbreeding of wild and escaped farm salmon may modulate the innate immune responses of the hybrid progeny compared with the wild parents. They also suggest that hybridization may not negatively impact the innate immune response in Atlantic salmon and therefore interbreeding resulting from escape events may not pose a threat to the innate immune response of wild populations.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degrees centigrade
µg	Microgram
µL	Microlitre
5loxb; alias *alox5b*	Transcript encoding Arachidonate 5-lipoxygenase b
ANOVA	Analysis of variance
baft3	Transcript encoding Basic leucine zipper ATF-like transcription factor 3
bp	Base pair
cathd	Transcript encoding Cathepsin D
cDNA	Complementary deoxyribonucleic acid
clrc	Transcript encoding C-type lectin receptor gene
CT	Cycle threshold
ctsf	Transcript encoding Cathepsin F
dsRNA	Double-stranded ribonucleic acid
dus6	Transcript encoding Dual specificity protein phosphatase
eif3d	Transcript encoding Eukaryotic translation initiation factor 3 subunit D
EO	European origin
Farm.EO	European farmed
Farm.NA	North American farmed
gDNA	Genomic deoxyribonucleic acid
g	Grams
gig1a	Transcript encoding Gig1 protein
GOI	Gene of interest

h	Hour
helz2; alias *PRIC285*	Transcript encoding Helicase with Zinc finger domain 2; Peroxisomal proliferator-activated receptor alpha-interacting cofactor complex, 285 KD subunit
herc3	Transcript encoding HECT and RLD domain containing E ubiquitin ligase 3
hpi	Hours post-injection
HSI	Hepatosomatic index
Hyb.EO $_{F♀}$	European farmed female crossed with wild male
Hyb.EO $_{W♀}$	Wild female crossed with European farmed male
Hyb.NA $_{F♀}$	North American farmed female crossed with wild male
if5	Transcript encoding Transcript encoding eukaryotic translation initiation factor 5
ifit5	Transcript encoding Interferon-induced protein with tetratricopeptide repeats 5
IFN	Interferon
ifna	Transcript encoding Interferon alpha
ifng	Transcript encoding Interferon-gamma
irf1a and *b*	Transcript encoding Interferon regulatory factor 1 paralogues a and b
irf3	Transcript encoding Interferon regulatory factor 3
irf7a and *b*	Transcript encoding Interferon regulatory factor 7 paralogues a and b
isg15a	Transcript encoding Interferon-stimulated gene 15a
IP	Intraperitoneal
IRF	Interferon regulatory factor

ISG	Interferon-stimulated gene
jak3	Transcript encoding Janus kinase 3
K	Fulton's condition factor
kg	Kilogram
L	Litre
dhx58; alias *lgp2*	Transcript encoding RNA helicase LGP2
m	Metre
mg	Milligram
min	Minute
mL	Millilitre
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
mxa and *b*	Transcript encoding Interferon-induced GTP-binding protein of myxovirus resistance paralogues a and b
NA	North American
ndufs7	Transcript encoding Ubiquinone oxioreductase core subunit S7
nf	Nuclear factor NF-kappa-B p100 subunit
ng	Nanogram
NRQ	Normalized relative quantity
NTC	No-template control
NTG	Non-transgenic
no-RT	No reverse transcriptase
p	P-value

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pgds	Transcript encoding Prostaglandin-D synthase
pIC	Polyriboinosinic polyribocytidylic acid
PIT	Passive integrated transponder
PRR	Pathogen recognition receptor
QC	Quality control
qPCR	Real-time quantitative polymerase chain reaction
RDA	Redundancy analysis
rpl32	Transcript encoding 60S ribosomal protein 32
RQ	Relative quantity
rsad2; alias *viperin*	Transcript encoding radical S-adenosyl methionine domain containing protein 2
SAV3	Salmonid alphavirus-subtype 3
SE	Standard error of the mean
socs1	Transcript encoding Suppressor of cytokine signaling 1
ssRNA	Single-stranded ribonucleic acid
stat1a, b and *c*	Transcript encoding Signal transducer and activator of transcription 1a, b and c
t_0	Time point 0
tgfb	Transcript encoding Transforming growth factor beta
tlr3	Transcript encoding Toll-like receptor 3
tlr7	Transcript encoding Toll-like receptor 7

traf5c	Transcript encoding Tumor necrosis factor receptor-associated factor 5 paralogue c
VSI	Viscerosomatic index

CO-AUTHORSHIP STATEMENT

The research described in this thesis was performed by Sindy L. Dove, with guidance from Drs. Matthew L. Rise and Ian A. Fleming. Sindy Dove was responsible for collecting and analysing data and writing the thesis. However, several others were instrumental in the completion of this research as well.

Shahin S. Islam (PhD candidate) was responsible for conducting the experiment for the manuscript in my absence, prior to the beginning of my masters. Dr. Mohamed Emam was responsible for assisting during tissue sample collection, troubleshooting and completion of qPCR. Dr. Sarah J. Lehnert provided guidance and training for statistical analysis, especially redundancy analyses, as well editing and providing feedback on manuscript. Eric H. Ignatz (PhD candidate) helped edit the manuscript and assisted in statistical analysis and interpretation. Drs. Albert Caballero-Solares and Khalil Eslamloo aided in conducting the experiment and provided training on RNA extraction, cDNA synthesis and qPCR analysis, as well as providing feedback on the manuscript. Drs. Ian Fleming and Matthew Rise supervised throughout the entire program, and both helped edit the thesis, as well as assisting with the tissue sample collection.

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1. INTRODUCTION

Aquaculture is an economically important and fast-growing food sector worldwide. Due to factors such as an abundance of clean, cold seawater, and established trade relationships worldwide, Canada has great natural advantages for marine aquaculture (Chopin, 2015). There are three major types of aquaculture: finfish, shellfish and seaweed (Chopin et al., 2012). Aquaculture is thought to be a necessary way to provide aquatic products for food consumption to be sustainable in the future (FAO SOFIA 2020 reviews). However, the aquaculture environment may often be very different from the natural environment, which can lead to domestication as the lifecycle occurs fully in captivity, independent of the wild environment (Liao and Huang 2000). Domesticated species, including but not limited to Atlantic salmon (*Salmo salar*), are often genetically different from their wild populations due to intentional and unintentional selection processes. For example, animals can be artificially selected for faster growth and disease resistance (Houston et al., 2020), and unintentional selection associated with captivity can result in the alteration of traits (reviewed in Glover et al., 2017) and loss of genetic variation (Karlsson et al., 2016). This leads to concerns about the impact of aquaculture escapees interbreeding with wild counterparts.

One of the major aquatic species farmed in Canada is Atlantic salmon. Atlantic salmon are ecologically, culturally and economically important. Typically an anadromous species, wild Atlantic salmon occupy rivers on both sides of the North Atlantic Ocean. Their freshwater range extends from the Connecticut River, U.S.A., to Ungava Bay, Canada, in the western Atlantic and from northern Portugal to the Barents Sea in the eastern Atlantic. However, the number of wild Atlantic salmon has declined in recent decades (Dadswell et al., 2021) and this has led to more reliance on aquaculture for commercial harvest. The first salmon breeding program involving

directed selection was in Norway and started in 1975 (Gjedrem 2010), however Atlantic salmon aquaculture began in the early 1970s (Gjøen and Bentsen 1997). Since 1970, the aquaculture of Atlantic salmon has grown to become an important economic industry across the globe, with the top five countries being Norway, Scotland, Faroe Islands, Chile and Canada (FAO, 2018). Strains of Atlantic salmon have been farmed for at least 15 generations (Gjedrem 2000; Gjedrem 2010), making them one of the most domesticated salmonid finfish species (Teletchea and Fontaine, 2014). Domestication of Atlantic salmon has led to many differences between wild and farmed salmon populations. For example, in aquaculture, production traits that have been selected for in Atlantic salmon include increased growth, late maturation, improved fillet quality, increased utilization of feed, and greater disease resistance (Thodesen et al., 1999; Gjedrem and Baranski, 2010; Solberg et al., 2013; Karlsson et al., 2016). Traits are also unintentionally selected for, especially behavioural traits that accompany selected traits such as growth (Huntingford, 2004). Such unintentional selection can also be from conditions of aquaculture, as behavioural biologists have identified many ways that conditions (e.g. farm raised) can effect trait selection (Bateson and Martin, 1999).

Given the differences between wild and farmed salmon, one of the concerns surrounding salmon aquaculture is the escape of farmed fish from net pens that can lead to intraspecific hybridization with wild populations, which are genetically unique from farmed populations, and disrupt the expression of fitness-related traits (Glover et al., 2017). The reported number of escaped salmon in the North Atlantic region is estimated at 2 million fish annually (Yeates et al., 2014; Keyser et al., 2018). Escape events are expected due to factors such as net changing and damage from storms and boat collisions (Thorstad et al., 2008; Jensen et al., 2010). In 2013, there was a large escape event of 20,000 Atlantic salmon in southern Newfoundland, Canada

(Wringe et al., 2018). The escaped salmon were mature and in spawning condition when entering the local streams and this led to hybridization on a broad scale with offspring of escapees found in 17 out of 18 rivers that were sampled (Wringe et al., 2018). This raises concerns for wild populations because in Newfoundland farmed salmon originate from the Saint John River, New Brunswick (NB), and are genetically distinct from the local populations (Wringe et al., 2018). Therefore, following an escape event, the local wild populations are at risk of genetic introgression that could alter naturally selected traits (Glover et al., 2017), reduce productivity, and lead to demographic decline (Hindar et al., 2006; Glover et al., 2017; Bradbury et al., 2020).

Recently, there has been an approval to import a Norwegian strain of salmon [Benchmark Genetics (StofnFiskur strain), Iceland] into Newfoundland to be farmed as triploids. Based on current knowledge of the triploidization process, the success rate of triploidization is approximately 98%, thus allowing for some of the population to be reproductively viable (Benfey, 2015). While the success rate of triploidization is high, even a small degree of ineffectiveness can be concerning when undertaking large-scale commercial production (Benfey, 2015). Therefore, the farming of Norwegian salmon in Newfoundland could introduce the new Norwegian strain to wild populations if an escape event and hybridization occur. In addition, recent work has demonstrated large genomic differences between Norwegian and North American Atlantic salmon (Lehnert et al. 2020); therefore, raising additional concerns about interactions of introduced Norwegian salmon with wild populations in Newfoundland.

Hybridization of domesticated and wild salmonids has been studied for over 25 years, however the immune response of the hybrids compared to their parent pure cross types is understudied. The likelihood of fitness reduction in hybrids could depend on the magnitude of differences between the parent cross types (Debes et al., 2012). Most genetic studies done thus

far show that hybrid salmon are intermediate to farmed and wild salmon for attributes such as lysosome activity (Johnson et al., 2003), and the expression of stress related genes (Bicskei et al., 2020). Another study done on three cross types of Atlantic salmon smolts (one wild, one domesticated and one hybrid) found that domesticated salmon showed a higher transcription level, of many genes including some immune relevant genes, compared to wild salmon (Debes et al., 2012). Contrary to the hypothesis of additive genetic effects resulting in hybrid salmon being intermediate for traits compared to farmed and wild parents, they found a mosaic of similarity to one parent or the other (Debes et al., 2012). Normandeau et al. (2009) also found that the consequence of hybridization between farmed and wild salmon on gene expression depends on population-specific genetic differences. Similar to my experiment, Normandeau et al. (2009) examined Atlantic salmon found in eastern Canada and created backcrosses to compare the wild, domesticated and hybrid salmon; however, their study did not include European Atlantic salmon. They concluded that the inheritance of gene expression patterns from a parent salmon to an offspring hybrid is highly variable (Normandeau et al., 2009).

Several studies of hybridization in domesticated fishes indicate strong parental inheritance of traits such as fitness, lysosome activity, growth performance, and stress related genes (Johnson et al., 2003; Panase and Mengumphan 2015; Bicskei et al., 2020). A study examining fitness traits in Atlantic salmon parr, compared farmed, native, and hybrid salmon (Einum and Fleming, 1997). This study showed that the hybrid salmon were intermediate to the farmed and wild salmon in both aggressiveness and risk aversion (Einum and Fleming, 1997). Johnson et al., 2003 found that in Chinook salmon smolts, genes encoding lysosome activity showed a strong sire component of heritability. A study done on catfish (*Pangasianodon gigas*) showed that the inheritance of growth performance traits was strongly maternal (Panase and

Mengumphan, 2015). Transcriptomics of Atlantic salmon fry showed that most effects of hybridization were additive (Bicskei et al., 2020). There were 18-32% of genes that showed maternal dominance, however this could have been due to the maternal environment effects studied (Bicskei et al., 2020). They also showed that there was paternal dominance in 11-15% of the genes (Bicskei et al., 2020). The inheritance patterns of traits from parents to hybrid offspring has shown substantial variability and could depend on many factors including parental environments as well as offspring environments. Thus far, information on the immune system is lacking for hybrid cross types, yet it is an essential aspect of overall fish health (Johnson et al., 2003).

The main aim of this study was to explore the impact of hybridization in Atlantic salmon on their innate antiviral-like immune response using molecular techniques. Many of the viruses that infect Atlantic salmon are known RNA viruses (Lang et al., 2009). The different types of RNA viral genomes are negative-sense single-stranded RNA [e.g. Infectious Salmon Anemia virus (ISAv) and Infectious Haematopoietic Necrosis virus (IHNv)], double-stranded RNA [e.g. Infectious Pancreatic Necrosis virus (IPNv)], and positive-sense single-stranded RNA viruses (e.g. members of family Nodaviridae) (Lang et el., 2009). These viruses are recognized by the host's pattern recognition receptors (PRRs) that initiate the innate immune response (Akira, Uematsu, and Takeuchi, 2006). These PRRs (i.e. *tlr7* and *tlr9*) are capable of recognizing pathogens by detecting pathogen-associated molecular patterns (PAMPS) such as dsRNA (Mogensen and Paludan, 2005). The main functions of PRRs include mediating the production of inflammatory cytokines, enhancement of phagocytosis, and maturation of dendritic cells (Mogensen and Paludan, 2005). Atlantic salmon aquaculture is impacted by various pathogenic viruses including many with RNA genomes (i.e. ISAv, togaviridae, and NSAV/SAV3), as

mentioned above (Lang et al., 2009). The salmonid aquaculture industry can experience great losses due to these viral infections (Collet, 2014), and there is a lack of effective commercial vaccines (Dhar et al., 2014). Many fish immunology researchers use polyriboinosinic polyribocytidylic acid [pIC, a synthetic double-stranded RNA (dsRNA) analogue of viral dsRNA, which induces interferons in vertebrates, like a PAMP (Akira et al., 2006)] in studies to elicit an innate antiviral-like immune response (Hori et al., 2012; Langevin et al., 2013; Caballero-Solares et al., 2017). Replication and symmetrical transcription in single-stranded RNA and DNA viruses, respectively, create dsRNA (Akira et al. 2006); pIC can serve as a useful proxy for studies of fish antiviral-like responses, and may lead to hypotheses regarding fish responses to viral pathogens.

Here, I used pIC to examine effects of hybridization on the antiviral-like innate immune response in Atlantic salmon. Wild salmon and both North American and European farmed salmon were used to create hybrid crosses and evaluate differences between pure and hybrid cross types. Using real-time quantitative polymerase chain reaction (qPCR) to measure expression of antiviral biomarker genes (e.g., *stat1, irf7, isg15, mx*), the impact of hybridization on Atlantic salmon response to intraperitoneal (IP) injection with pIC (Caballero-Solares et al., 2017; Eslamloo et al., 2017), compared with the phosphate buffered saline (PBS)-injected controls was determined. The innate antiviral biomarker genes were chosen to represent the interferon pathway of the innate immune response specifically to the IP injection of pIC (Eslamloo 2017, 2018). All genes selected for testing were chosen based on known responses of Atlantic salmon to pIC injection (Eslamloo et al., 2017, Caballero-Solares et al., 2017).

I inferred that this study would add to our understanding of the potential consequences of escape events and hybridization on wild-farm hybrid salmon immune responses, and increase the

understanding of how the innate immune system differs among cross types of Atlantic salmon. It was hypothesized that the hybrid cross types would show intermediate responses compare to the pure farmed and wild cross types. Where some farmed species have been bred for immune resistance (Gjedrem and Baranski, 2010), it was hypothesized that the farmed cross types would show a stronger expression of the chosen innate immune-relevant genes in response to pIC than the wild cross type. The information gained from this study will lead to the formation of hypotheses regarding the potential consequences of interbreeding of wild and escaped farm salmon on the innate immune responses of the hybrid progeny.

2. MATERIALS AND METHODS

2.1 Fish Crosses

Six different types of crosses between farmed and wild Atlantic salmon (Figure 2-1) were created between November 20 and December 5, 2015 (see Islam et al., 2020), at Memorial University's Ocean Sciences Centre (Logy Bay, NL, Canada). There were 76 families generated in total that were pooled by cross type at the start of exogenous feeding (fry stage), to compose the groups in this study and reared until the age of 2.5 years. The parents for each cross type came from one of three origins: 1) North American farmed, originating from the Saint John River, NB, but farmed across Atlantic Canada; 2) European farmed from the Icelandic Benchmark Genetics facility (Benchmark Genetics has three strains of salmon, and we used their StofnFiskur strain); and 3) wild from Northeast Placentia River, NL. The specific wild population was included in this study because it shows evidence of EO introgression from when glaciers retreated (~1% of genome; I.R. Bradbury personal communication), and salmon recolonized approximately 10,000 years ago (Bradbury et al. 2015). The six cross types used in this study were: pure North American farmed (Farm.NA; 20 families), pure wild (Wild; 11 families), pure European farmed (Farm.EO; 10 families), Wild female crossed with Farm.EO male ($Hyb.EO_{W♀}$; 10 families), Farm.EO female crossed with Wild male ($Hyb.EO_{F♀}$; 12 families), and Farm.NA female crossed with Wild male ($Hyb.NA_{F♀}$; 13 families) (for cross type details see Islam et al., 2020). Hybrid abbreviations have the maternal parent denoted in subscript where W♀ is Wild dam, and F♀ is Farmed dam.

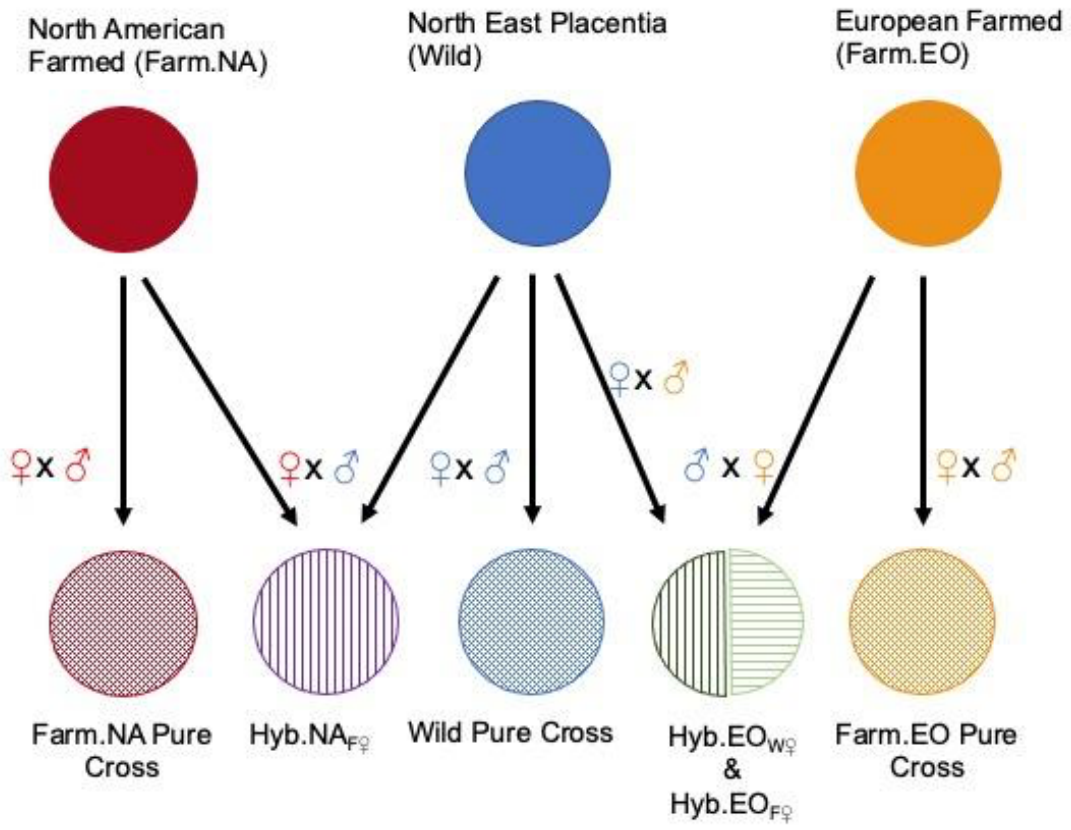

Figure 2-1. Cross type generation design among North American farmed (Farm.NA), Wild, and European farmed (Farm.EO) salmon, to generate 6 cross types (bottom left to right): pure North American farmed (Farm.NA), Farm.NA female crossed with wild male (Hyb.NA $_{F♀}$), pure wild (Wild), wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with wild male (Hyb.EO $_{F♀}$), and pure European farmed (Farm.EO).

2.2 Fish Handling and Sampling

The salmon were reared in a freshwater flow-through tank system from the start of exogenous feeding for this experiment. Fish were fed EWOS salmon feed (EWOS-Cargill, BC, Canada) of pellet size ranging from crumble (0.5 g; 55% protein and 15% fat) to size 2 (2 mm; 50% protein and 20% fat), as they grew to the size of experimental fish. Prior to the current research, salmon were kept at ambient water temperature, which varied seasonally (10^{o}C ± 7^{o}C), and air saturation was maintained at 90% ± 5%. During experimentation (March 12, 2018 – April 26, 2018), fish [64.5 ± 18.9 g SD (Table 2-1)] were reared at ~6 ^{o}C, ≥ 95% air saturation, and 12L: 12D photoperiod. Six weeks prior to experimentation (March 12, 2018), 80 fish per cross type were tagged with passive integrated transponders [PIT (Biomark Inc., Boise, ID, USA)] to uniquely identify individual fish. The fish were placed into 6 tanks (475 L water capacity) at a density of 10.86 kg.m^{-3}, to separate sampling time points. Three tanks were used for 24 h post-injection (hpi) and three tanks used for 72 hpi, with two cross types combined per tank (Figure 2-2). Both PBS and pIC injected fish were placed in the same tanks and identified with PIT tags upon sampling.

Table 2-1. Weight, length, Fulton's condition factor (K), hepatosomatic index (HSI), and viscerosomatic index (VSI) of Atlantic Salmon (n=10), of six different cross types. Cross type abbreviations: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), Wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with Wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with Wild male (Hyb.NA $_{F♀}$).

Variables	Cross Type											
	Wild		Farm.EO		Farm.NA		Hyb.EO$_{W♀}$		Hyb.EO$_{F♀}$		Hyb.NA$_{F♀}$	
	Mean	(± SE)	Mean	(± SE)	Mean	(± SE)	Mean	(± SE)	Mean	(± SE)	Mean	(± SE)
Weight (g)	72.85	1.92	61.10	2.41	89.28	2.78	68.14	2.62	60.12	2.08	85.32	2.54
Length (cm)	19.42	0.16	17.98	0.21	20.09	0.18	18.69	0.22	18.19	0.19	19.94	0.19
K	0.98^{b}	0.01	1.02^{ab}	0.01	1.09^{a}	0.01	1.02^{ab}	0.01	0.98^{b}	0.01	1.07^{ab}	0.02
HSI (%)	1.17	0.03	1.13	0.03	1.03	0.02	0.97	0.03	1.10	0.02	1.00	0.04
VSI (%)	8.07	0.14	7.31	0.12	7.75	0.12	7.43	0.20	7.67	0.16	7.50	0.19

Mean values are given with their standard errors (± SE). Superscripted lower-case letters denote significant differences between cross types ($p < 0.05$).

Before immune stimulation or handling (April 24, 2018), ten fish per cross type were taken from the holding tanks before the rest of the fish were moved to the experimental tanks. They were then euthanized with an overdose of tricaine methanesulfonate (MS-222; 400 $mg.L^{-1}$, AQUALIFE TMS, Syndel Laboratories Ltd., Nanaimo, BC, Canada) buffered with sodium bicarbonate, and head kidney tissues were sampled and flash-frozen in liquid nitrogen then stored at -80°C, at time 0 (t_0) for basal measurements of gene expression. The remaining salmon were intraperitoneally (IP) injected with either polyriboinosinic polyribocytidylic acid [pIC; 2 $\mu g.g^{-1}$ of fish (Sigma-Aldrich, Oakville, ON, Canada; Catalog #42424-50-0)], a synthetic double-stranded RNA (dsRNA) that elicits a potent innate antiviral-like immune response related to viral infection (Akira et al., 2006), or an equal volume of sterile phosphate buffered saline [PBS (Thermo Fisher Scientific, Mississauga, ON, Canada)] to serve as a mock-injection control. Each injection group had 40 fish per cross type. For all injections, the salmon were starved for 24 h and lightly anesthetized using MS-222 (50 $mg.L^{-1}$) buffered with an equal amount of sodium bicarbonate. During this time, fish were taken from their original tanks and placed into the new holding tanks, combining pure crosses with their related maternal hybrids, for the remainder of the experiment (Figure 2-2).

At 24 and 72 hpi, fish (n = 10) were euthanized as previously described; head kidney samples were collected using standard aseptic techniques, flash-frozen in liquid nitrogen, and were stored at -80°C until RNA extractions could be performed.

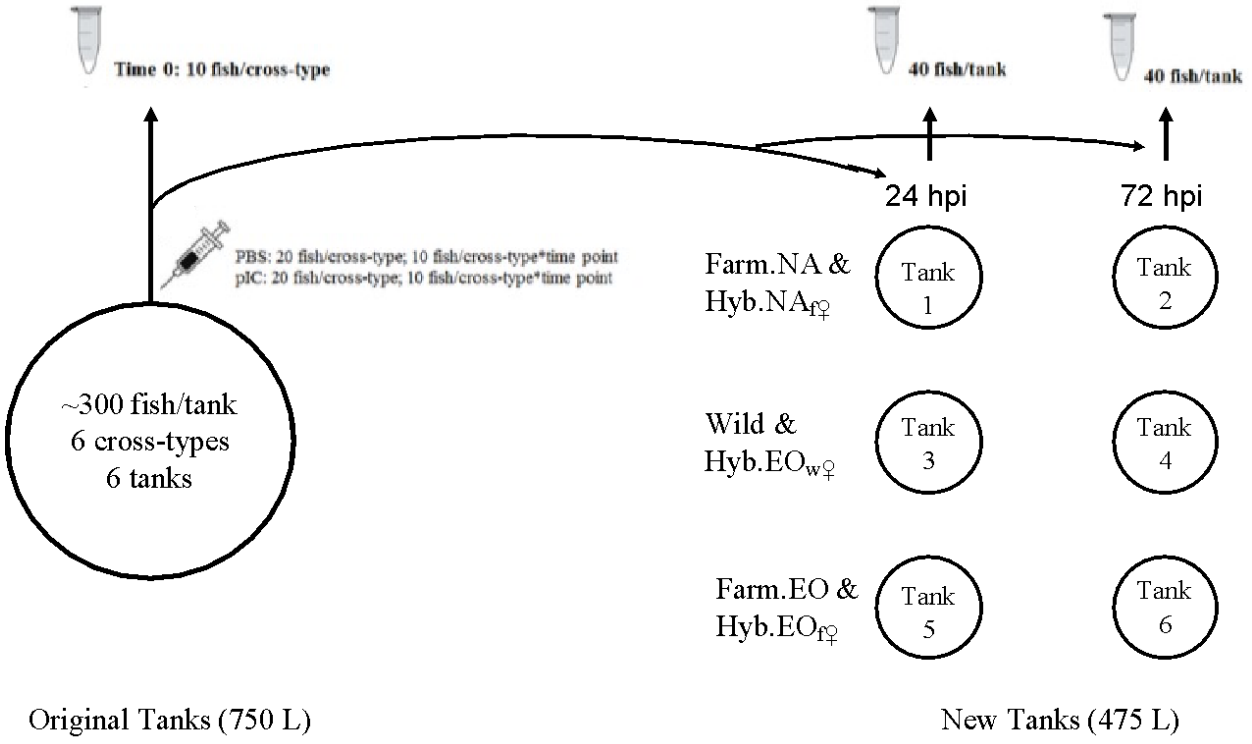

Figure 2-2. Experimental design for sampling Atlantic salmon IP injected with either pIC or PBS (control). Cross-type abbreviations in legend: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with wild male (Hyb.NA $_{F♀}$).

2.3 RNA extraction and purification

For extraction, head kidney tissue (~100 mg) was homogenized using a motorized Kontes RNase-free pellet pestle grinder (Kimble Chase, Vineland, NJ) in 800 µL of TRIzol (Invitrogen, Carlsbad, CA, USA). The tissue was further processed using QIAshredder (QIAGEN, Mississauga, ON, Canada) columns. Residual genomic DNA was degraded using DNase I (QIAGEN), and RNA samples were further purified using RNeasy MinElute Cleanup Kits (Invitrogen, Burlington, ON, Canada). All tissue processing, RNA extraction and purification procedures were performed according to the manufacturers' protocols. RNA integrity and lack of genomic DNA contamination was verified using 1% agarose gel electrophoresis, prepared using 1X TAE and ethidium bromide (0.5 $\mu g.mL^{-1}$) and imaged on a SynGene G:Box gel imager (Figure 2-3). The size of total RNA bands was determined compared to a 1 kb+ ladder (Invitrogen, Carlsbad, CA, USA). The purity was assessed using absorbance at the 260 nm wavelength divided by absorbance at the 280 nm wavelength (A260/280) and absorbance at the 260 nm wavelength divided by absorbance at the 230 nm wavelength (A260/230) NanoDrop UV spectrophotometry (Table 2-2). Only high-quality samples with distinct 18S/28S ribosomal RNA (rRNA) bands and acceptable A260/230 (> 1.85) and A260/280 (>2.00) were used in complementary DNA (cDNA) synthesis reactions for qPCR. The gel images for all samples were comparable in total RNA quality and all samples showed similar high quality, clear rRNA bands depicting high quality RNA going forward to cDNA synthesis.

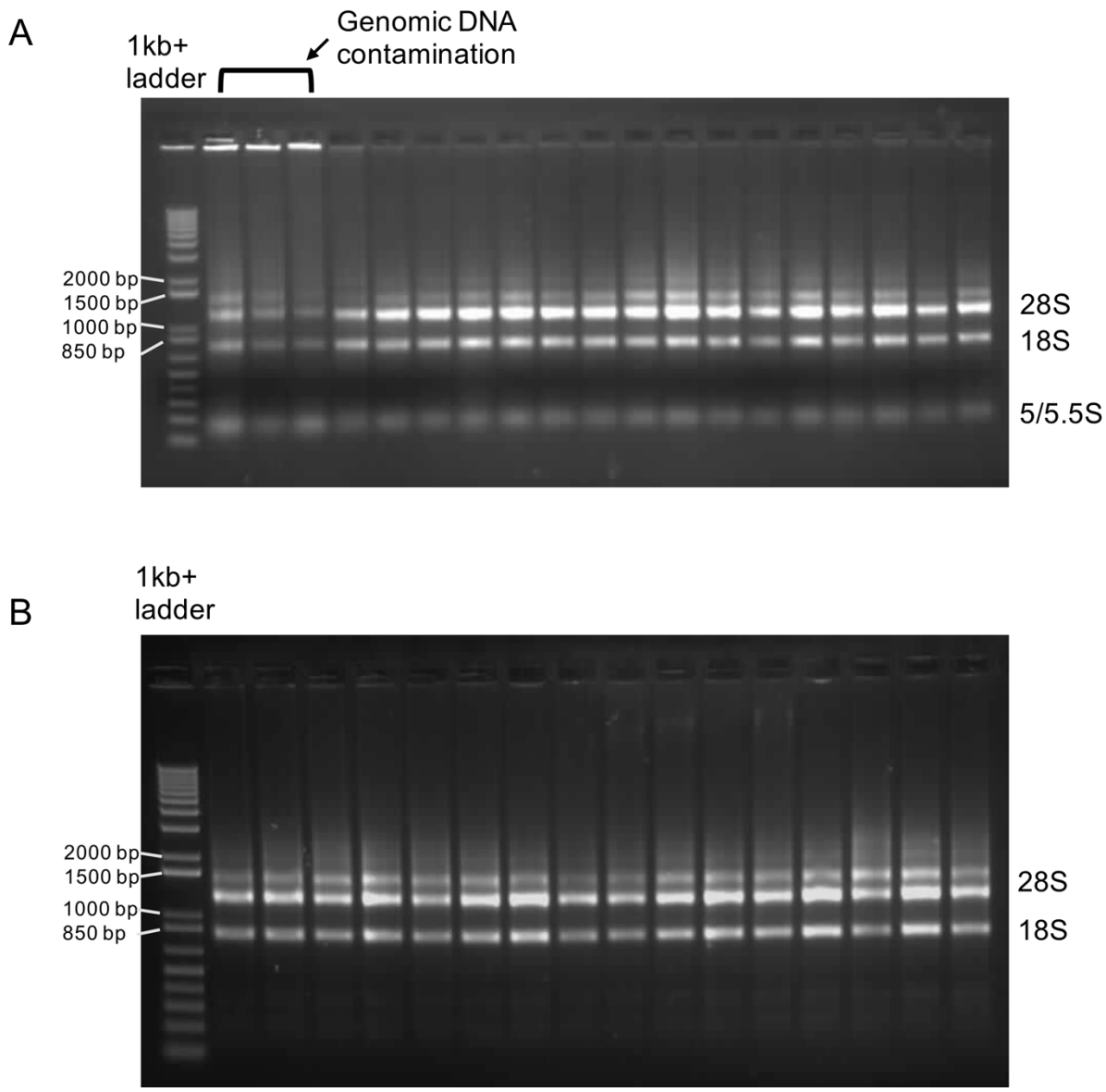

Figure 2-3. Examples of agarose gels (1%) comparing A: total RNA samples prior to DNase digestion and MinElute purification and B: total RNA samples after DNase digestion and MinElute purification. A subset of representative total RNA samples are shown, with 28S and 18S ribosomal RNA (rRNA) bands depicting RNA quality. Samples in gels from well 2-20: Hyb.NA $_{F♀}t_0$, Hyb.NA $_{F♀}$ t_0, Hyb.EO $_{W♀}t_0$, Hyb.EO $_{W♀}t_0$, Hyb.EO $_{F♀}t_0$, Hyb.EO $_{F♀}t_0$, Wild t_0, Hyb.NA $_{W♀}$PBS 72 hours post-injection (hpi), Hyb.EO $_{W♀}t_0$, Hyb.EO $_{W♀}t_0$, Hyb.EO $_{F♀}t_0$, Hyb.EO $_{F♀}t_0$, Farm.NA t_0, Farm.NA PBS 24 hpi, Farm.NA PBS 24 hpi, Hyb.EO$_{W♀}$ pIC 24 hpi, Hyb.EO $_{F♀}$PBS 24 hpi, Hyb.EO $_{F♀}$PBS 24 hpi, Farm.Na PBS 24 hpi. B is missing samples Hyb.EO $_{F♀}$PBS 24 hpi, Hyb.EO $_{F♀}$PBS 24 hpi, Farm.Na PBS 24 hpi, that are in wells 18-20 in A. Cross type abbreviations: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with wild male (Hyb.NA $_{F♀}$).

Table 2-2. Extracted head kidney RNA pre-cleaned and post-cleaned A260/280 and A260/230 ratios for all samples. Cross type abbreviations: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with wild male (Hyb.NA $_{F♀}$).

Time Point	t_0				PBS 24 hpi				pIC 24 hpi				PBS 72 hpi				pIC 72 hpi			
	Pre-cleaned		Post-cleaned		Pre-cleaned		Post-cleaned		Pre-cleaned		Post-cleaned		Pre-cleaned		Post-cleaned		Pre-cleaned		Post-cleaned	
Sample	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230
1 Wild	1.98	0.94	2.10	2.34	2.10	2.27	2.14	2.32	2.08	2.20	2.09	2.29	2.02	1.98	2.05	2.35	2.03	2.03	2.07	2.25
2 Wild	2.08	1.77	2.15	2.26	2.07	1.78	2.11	2.32	2.07	1.96	2.15	2.13	2.00	1.83	2.12	2.29	2.03	1.88	2.09	2.31
3 Wild	2.08	1.65	2.12	2.40	2.10	2.02	1.81	1.77	2.07	1.91	2.17	2.31	2.00	1.87	2.13	1.62	2.03	1.80	2.08	2.32
4 Wild	2.05	1.59	2.03	2.30	2.07	1.89	2.16	2.30	2.05	2.14	2.12	2.26	1.98	1.38	2.11	2.32	2.02	1.34	2.01	2.26
5 Wild	2.06	1.79	2.16	2.33	2.06	2.01	2.14	2.35	2.03	1.93	2.08	2.37	2.00	1.49	2.17	2.31	2.01	1.29	2.12	2.31
6 Wild	2.03	1.59	2.16	2.34	2.03	1.64	2.12	2.29	1.96	1.25	2.06	2.23	1.88	1.39	2.11	2.34	2.01	1.51	2.13	2.33
7 Wild	2.02	1.29	2.14	2.32	2.03	1.74	2.10	2.37	1.99	1.21	2.15	2.30	1.94	1.07	1.99	2.38	1.97	1.36	1.80	1.72
8 Wild	2.03	2.05	2.00	2.29	2.04	1.67	2.09	2.34	1.99	1.46	2.13	2.27	2.00	1.47	2.10	2.34	2.00	1.43	2.14	2.12
1 Farm.EO	2.10	2.18	2.16	2.29	2.07	1.79	2.10	2.31	2.04	2.16	2.07	2.15	2.06	2.02	2.13	2.28	2.06	2.05	2.10	2.28
2 Farm.EO	2.04	1.18	2.13	2.37	1.99	1.42	2.13	2.29	2.04	1.41	2.08	2.28	2.04	1.91	2.01	2.21	2.08	2.21	2.07	2.30
3 Farm.EO	2.06	1.65	1.95	1.90	2.00	1.54	2.14	1.78	1.98	1.36	2.11	2.29	2.05	1.32	2.13	2.26	2.06	1.85	2.02	2.21
4 Farm.EO	2.03	1.25	2.10	2.33	2.03	1.41	2.14	2.07	2.00	1.54	2.03	2.28	2.01	1.51	2.16	2.08	2.19	1.74	2.07	2.31
5 Farm.EO	2.04	1.57	2.02	2.34	2.03	1.56	2.19	2.13	2.00	1.37	1.98	2.30	1.94	0.84	2.13	2.32	2.00	1.16	2.16	2.30
6 Farm.EO	2.01	1.74	2.13	2.35	2.03	1.29	2.07	2.33	2.01	1.46	2.09	2.32	2.00	1.29	2.12	2.31	2.01	1.34	2.14	2.36
7 Farm.EO	2.05	1.45	2.06	2.30	1.99	1.01	2.12	2.26	2.02	1.43	2.09	2.32	2.02	1.45	2.09	2.33	1.99	1.39	1.79	1.63
8 Farm.EO	2.04	1.75	2.14	2.31	2.02	1.56	2.13	2.30	1.99	1.32	2.16	2.25	2.01	1.23	2.13	2.35	2.01	1.53	2.02	2.31
1 Farm.NA	2.09	1.88	2.11	2.32	2.07	1.94	2.16	2.23	2.05	1.57	1.98	2.21	2.03	1.38	2.09	2.40	2.08	1.72	2.12	2.33
2 Farm.NA	1.97	1.01	2.14	2.35	1.91	1.63	2.14	2.32	2.08	1.87	2.09	2.22	2.06	1.80	2.12	2.34	2.03	1.54	2.11	2.20
3 Farm.NA	2.09	1.99	2.11	2.38	2.05	2.07	2.14	2.23	2.07	1.73	2.13	2.22	2.01	1.16	2.07	2.35	2.02	1.58	2.12	2.33
4 Farm.NA	2.04	1.61	2.10	2.35	2.07	1.63	2.17	2.29	2.06	1.94	2.13	2.29	1.87	1.32	2.07	2.26	2.01	1.49	2.11	2.32
5 Farm.NA	1.94	1.36	2.15	2.31	2.01	1.50	1.94	2.04	2.08	1.89	2.17	2.33	2.00	1.35	2.08	2.31	1.99	1.40	2.12	2.25
6 Farm.NA	2.05	1.55	1.80	1.64	2.04	1.55	2.17	2.30	2.01	1.39	2.13	2.33	1.88	1.31	2.13	2.35	2.02	1.38	2.12	2.32
7 Farm.NA	2.04	1.52	2.16	2.26	2.04	1.59	2.16	2.26	1.99	1.38	2.12	2.31	2.02	1.44	2.13	2.32	1.99	1.26	2.12	2.32
8 Farm.NA	2.05	1.79	2.12	2.29	2.04	1.65	2.16	2.29	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.02	1.50	2.12	2.34
1 Hyb.EO$_{W}$♀	2.09	1.91	2.17	2.36	2.00	2.00	2.13	2.35	2.02	1.31	2.08	2.32	2.08	2.06	2.12	2.15	2.06	1.88	2.09	2.34
2 Hyb.EO$_{W}$♀	2.08	1.93	2.18	2.33	2.03	1.24	2.14	2.32	2.03	1.54	1.97	2.30	2.07	2.13	2.11	2.21	2.05	1.97	2.13	2.35
3 Hyb.EO$_{W}$♀	2.07	1.96	2.12	2.30	2.00	1.32	2.16	2.29	2.04	2.03	1.99	2.21	2.07	2.00	2.15	2.32	2.03	2.08	2.12	2.36
4 Hyb.EO$_{W}$♀	2.05	1.71	2.09	2.34	2.01	1.41	2.08	2.31	1.96	1.31	2.12	2.32	2.07	1.98	2.14	2.30	2.02	1.67	2.14	2.30
5 Hyb.EO$_{W}$♀	2.01	1.06	2.11	2.07	2.01	1.41	2.15	2.25	1.97	1.53	2.12	2.23	2.04	1.94	2.14	2.33	2.03	1.69	2.00	2.29
6 Hyb.EO$_{W}$♀	2.04	1.75	2.10	2.33	2.00	1.29	2.12	2.30	2.02	1.56	2.17	2.28	2.02	1.98	2.12	1.80	2.02	1.65	2.09	2.34
7 Hyb.EO$_{W}$♀	2.06	1.76	2.16	2.32	1.98	1.12	2.11	2.23	1.96	1.20	2.12	2.28	2.03	1.43	2.14	2.30	2.03	1.45	2.08	2.34
8 Hyb.EO$_{W}$♀	2.00	1.56	2.16	2.31	1.95	1.99	2.17	2.33	1.98	1.33	2.15	2.21	1.96	0.79	2.15	2.27	2.02	1.47	2.11	2.06
1 Hyb.EO F♀	2.08	1.97	2.15	2.35	2.08	2.01	2.14	2.29	2.07	1.96	2.15	2.26	2.07	2.04	2.08	2.39	2.07	2.06	2.11	2.31

Table 2-2 continued.

Time Point	t_0				PBS 24 hpi				pIC 24 hpi				PBS 72 hpi				pIC 72 hpi			
	Pre-cleaned		Post-cleaned		Pre-cleaned		Post-cleaned		Pre-cleaned		Post-cleaned		Pre-cleaned		Post-cleaned		Pre-cleaned		Post-cleaned	
Sample	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230	260/280	260/230
2 Hyb.EO F♀	2.05	1.33	2.13	2.33	2.06	1.91	2.12	2.32	2.06	1.60	2.16	2.31	2.09	2.22	2.13	2.09	2.07	1.68	2.13	1.79
3 Hyb.EO F♀	2.07	1.91	2.11	2.32	2.04	1.83	2.12	2.33	1.94	2.11	1.78	1.63	2.02	1.55	2.09	2.39	2.03	1.63	2.12	2.33
4 Hyb.EO F♀	2.08	1.89	2.15	2.34	2.04	1.49	2.00	2.26	2.04	2.01	2.13	2.28	2.01	1.17	2.07	2.39	2.01	1.43	2.11	2.34
5 Hyb.EO F♀	2.05	1.36	2.16	2.34	2.06	1.68	2.13	2.30	1.99	1.32	2.09	2.35	1.99	1.40	2.11	2.29	2.00	1.35	2.12	2.10
6 Hyb.EO F♀	2.03	1.64	2.16	2.30	2.05	1.55	2.14	2.28	1.98	1.43	2.11	2.31	2.02	1.38	2.11	2.33	2.02	1.55	2.14	2.06
7 Hyb.EO F♀	2.02	1.44	2.16	2.32	2.05	1.68	2.15	2.09	1.99	1.31	2.17	1.78	1.97	0.90	2.17	2.30	1.96	1.18	2.10	2.35
8 Hyb.EO F♀	2.03	1.45	2.11	2.31	2.06	1.73	2.16	2.33	1.99	1.39	2.14	2.27	1.97	0.93	2.11	2.35	2.02	1.49	2.11	2.34
1 Hyb.NA F♀	2.07	1.93	2.18	1.74	2.07	1.76	2.12	2.32	2.07	2.08	2.10	2.31	2.09	2.02	2.13	2.29	2.07	1.91	2.10	2.32
2 Hyb.NA F♀	2.03	2.04	2.15	2.34	2.09	1.95	2.15	2.30	2.08	1.73	2.12	2.32	2.09	1.95	2.12	2.28	2.09	2.00	2.08	2.27
3 Hyb.NA F♀	2.04	1.84	2.11	2.35	2.06	1.95	2.19	1.98	2.06	2.10	2.11	2.24	2.09	1.74	2.10	2.36	2.08	1.96	2.10	2.31
4 Hyb.NA F♀	2.03	1.30	2.11	2.32	2.06	7.87	2.14	2.19	2.01	1.27	2.14	2.27	2.07	1.80	2.15	2.31	2.05	1.80	2.03	2.28
5 Hyb.NA F♀	2.06	1.81	2.16	2.30	2.03	1.73	2.02	2.27	2.00	1.51	2.14	2.29	2.06	1.96	2.16	2.31	2.04	1.88	2.16	2.34
6 Hyb.NA F♀	2.05	1.69	2.12	2.31	2.05	1.67	2.05	2.28	2.02	1.56	2.14	2.25	2.05	2.03	2.15	1.89	2.01	1.48	2.14	2.31
7 Hyb.NA F♀	2.00	1.21	2.14	2.31	1.86	1.71	2.17	2.28	2.04	1.54	2.13	2.23	2.02	1.40	2.01	2.28	2.01	1.46	2.10	2.31
8 Hyb.NA F♀	1.96	0.94	2.05	2.24	2.00	1.77	2.15	2.28	2.01	1.50	2.13	2.38	2.03	1.69	2.16	2.25	2.02	1.46	2.14	2.26

A = absorbance
260, 280, 230 = wavelength in nanometers (nm)
t_0 = time zero, before treatment
hpi = hours post-injection
PBS = phosphate buffered saline (control injection)
pIC = polyriboinosinic polyribocytidylic acid

2.4 cDNA Synthesis

First-strand cDNA templates for qPCR were synthesized in 20 µl reactions by reverse transcription of 1 µg of DNase I-treated, column-purified total RNA using an iScript cDNA synthesis kit (Bio-Rad, Saint-Laurent, QC, Canada) following the manufacturer's protocol. Random primers (250 ng; Invitrogen/Life Technologies) and M-MLV reverse transcriptase (200 U; Invitrogen/Life Technologies) were used with first-strand buffer (1X final concentration), dNTPs (0.5 mM final concentration) and DTT (10 mM final concentration) at 37ºC for 50 min as per the manufacturer's protocols. Pooled cDNA was made to test primer pair quality. Three pools (Wild, Farm.EO and Farm.NA) were made consisting of four samples of 2 µg from each pIC 24 hpi Wild, pIC 24 hpi Farm.EO, and pIC 24 hpi Farm.NA, respectively. Lastly, a linker sample, composed of cDNA made from pooling equal concentrations from 2 samples (1 Wild PBS 72 hpi sample and 1 Hyb.EO $_{W♀}$ pIC 24 hpi sample), with the highest concentration, were made to test inter-plate variability, and used on every plate in this qPCR study. If the linker samples had a cycle threshold (CT) difference of more than 0.5 cycle, the plates were repeated. On average, the CT difference between linker samples on both plates for a given gene was 0.18 cycle. Aliquots of cDNA were prepared in amounts to be used per day and stored at -80ºC, to minimize freeze-thaw events during qPCR.

2.5 qPCR

There were 39 genes tested and 17 genes chosen to go forward. Primer pairs that were chosen to go forward had to meet the following criteria: pass primer quality testing (i.e. single melt curve peak; no primer dimer in the no-template controls (NTCs); amplification efficiency between 80% and 110%); and have at least a 2-fold difference between cross types for at least

one cross type pair (Table 2-3). The 17 genes chosen to go forward were also chosen to include at least 1 representative gene of three important aspects of the immune cellular response to pIC (Eslamloo et al., 2017): PRRs, signal transduction and effectors (Table 2-4). These three aspects were chosen because of their importance to the innate immune cellular response specifically to IP injected pIC (Hori et al., 2013; Eslamloo et al., 2016, 2017). A key part if the innate immune response is pathogen detection which is done by PRRs (Hori et al., 2012) that are encoded as a result of gene products from the interferon (IFN) pathway, that is the first line of defense against viral infections (Sadler and Williams (2008). These then activate signaling pathways which induce the innate immune response (Hori et al., 2012). PRR-encoding genes and signalling protein (e.g., *jak-stat*) encoding genes also work inside the nucleus and defend against the infection (Eslamloo et al., 2017). Therefore, these three aspects cover the host-pathogen interaction, the signal transduction and transcription of the virus, and the defense once inside the nucleus.

Paralogues are two or more genes that are derived from the same ancestral gene (Lien et al., 2016). These are important to this study because of a whole genome duplication event that happened in salmonids about 80 million years ago, resulting in the genome being recognized as pseudotetraploid (Lien et al., 2016). The decision to keep paralogues that responded differently in this study was made to incorporate the potential divergence between European and North American salmon after the whole genome duplication event (Lien et al., 2016). However, the divergence between North American and European salmon populations happened much more recently, about 1,670,000 years ago (Rougemont and Bernatchez, 2018). It has also been said to be >1,000,000 years ago (Nilsson et al., 2001) and as recent as 600, 000 – 700,000 years ago (King et al., 2007). These populations and the divergence between them are important to this

study due to the interest in farming European Atlantic salmon in North American farms. The paralogues that were retained differed in their responses, and if both paralogues responded similarly and had at least a two-fold change difference between cross types, only one was retained for analysis.

Primer pairs were quality control (QC) tested using three pools of cDNA in technical triplicates. The three pools selected for QC were Wild, Farm.EO, and Farm.NA as it was thought that the pure crosses would represent the extremes and the hybrid cross types would be intermediate. Five-point, three-fold dilution series were used to generate standard curves, the slopes of which were used to calculate percent amplification efficiency using the equation from Pfaffl (2001): $E = (10^{[-1/slope]}) *100$. This was performed in 13 µL reactions using 1X power SYBR Green PCR Master Mix (Bio-Rad), 50 nM of both the forward and reverse primers and 4 µl of diluted cDNA (5 ng input of total RNA). Using a ViiA7 RT-PCR (Real Time-PCR) System (Applied Biosystems/Life Technologies, Foster City, CA), qPCR amplification program for all primer pairs consisted of 1 cycle at 50 ^{o}C for 2 min., 1 cycle at 95 ^{0}C for 10 min., and 40 cycles of 95 °C for 15 sec. and 60 °C for 1 min. with data collection after each 60 °C step. Amplification curves and dissociation (i.e. melt) curves (Figure 2-4) were also used to assess qPCR primer pair quality. Final amplification efficiencies are reported as averages of the tested cDNA pools (Table 2-5). These criteria ensure that the qPCR assays performed comparably well with all population templates tested (Table 2-3).

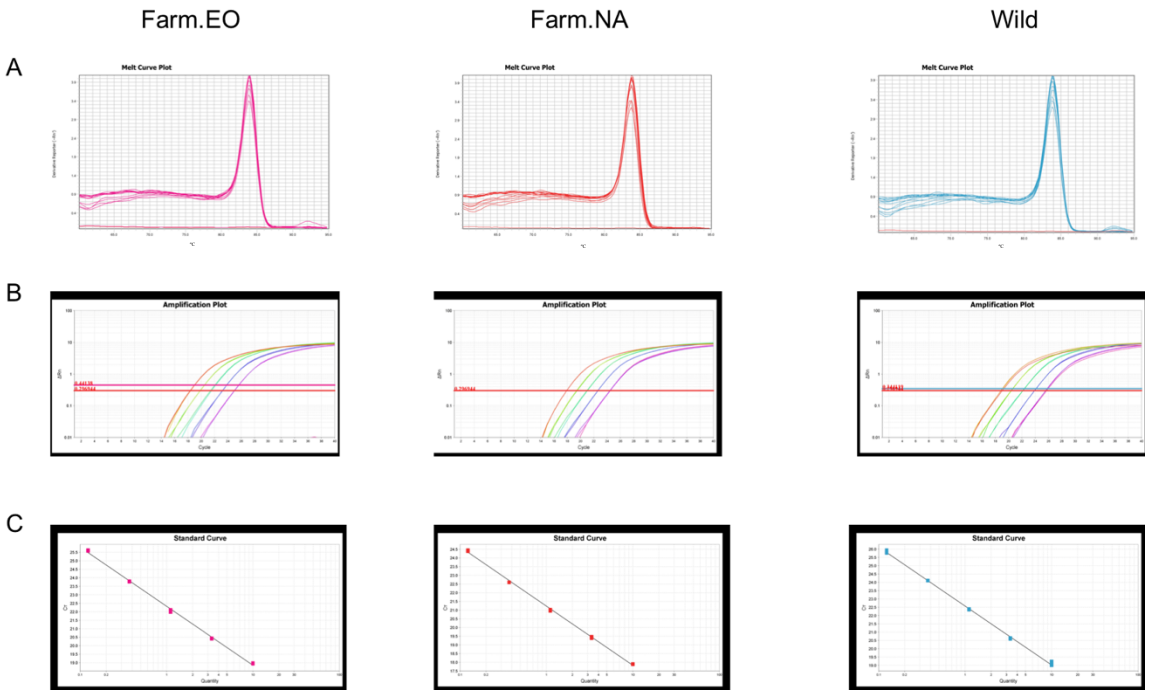

Figure 2-4. Example of results for *ifna* for parameters considered in qPCR primer pair quality testing: melt curve (A), amplification (B) and standard curve (C) when choosing normalizers and genes of interest, tested on three groups, European farmed (Farm.EO), North American farmed (Farm.NA), and Wild. For panels A and C, the colour represents the cross type. For panel B the colours represent the different dilutions (as described above in methods).

Table 2-3. Fold-change difference between pure cross types for primer quality control (QC) check to choose genes of interest. Cross type abbreviations: pure Wild, pure European farmed (Farm.EO) and pure North American farmed (Farm.NA).

Target	Farm.EO pIC-Farm.NA pIC	Farm.EO pIC-Wild pIC	Farm.NA pIC-Wild pIC	Farm.EO PBS-Farm.NA PBS	Farm.EO PBS-Wild PBS	Farm.NA PBS-Wild PBS	Passed QC
batf3-2	2.68	2.61	-0.06	-0.51	-0.05	0.46	N
cathd a	0.80	0.56	0.69	1.25	0.59	0.48	N
cathd b	0.84	0.52	0.61	1.11	0.63	0.57	N
cathd c	0.84	0.76	0.90	1.38	1.08	0.78	N
clrc	1.13	0.48	0.42	0.73	0.68	0.92	N
ctsf-1	2.28	2.07	-0.21	-0.98	-0.17	0.81	N
dus6-2	2.22	2.13	-0.09	-0.15	0.65	0.80	N
gig1 a-1	4.62	2.43	0.53	1.94	1.80	0.93	Y
gig1 b-2	0.69	0.32	0.46	1.49	1.00	0.68	N
herc3-1	2.34	2.05	-0.30	-0.25	-0.35	-0.11	N
ifit5	1.75	2.52	1.44	0.92	1.21	1.31	N
ifna	2.00	0.89	0.45	0.99	1.20	1.21	Y
ifng	2.82	1.60	0.57	1.04	0.91	0.88	Y
irf1a	2.30	1.97	0.86	1.25	1.27	1.02	Y
irf1b	2.46	2.09	0.85	1.45	1.54	1.06	N
irf3	2.12	1.40	0.66	1.55	1.86	1.20	Y
irf7a	1.69	1.27	0.75	1.35	1.53	1.13	N
irf7b	1.39	1.07	0.77	0.86	1.13	1.32	Y
isg15a	2.03	0.77	0.38	0.58	0.82	1.41	Y
isg15b	3.22	1.73	0.54	2.08	2.42	1.17	N
itif5-1	3.94	4.67	0.73	-1.06	0.29	1.35	N
jak3-1	3.06	3.27	0.21	0.03	0.55	0.52	N
dhx58	2.19	1.54	0.70	1.67	1.89	1.13	Y
mxa-1	1.08	0.74	0.68	0.79	0.89	1.12	N
mxb-2	6.69	8.58	1.28	1.36	1.98	1.46	Y
ndufs7	1.51	0.59	0.39	1.88	1.39	0.74	N
nf-1	1.38	1.85	0.47	-0.15	0.47	0.62	N
rsad2a-1	2.96	3.72	1.25	1.19	2.37	1.99	Y
rsad2b-2	2.05	3.30	1.61	1.88	2.27	1.20	Y
socs1-2	1.40	1.63	0.23	-0.60	0.17	0.77	N
stat1a	2.00	1.32	0.66	1.25	1.45	1.16	Y
stat1b	1.45	1.11	0.76	0.83	1.13	1.35	N
stat1c	1.44	1.25	0.86	0.99	1.74	1.76	Y
tgfb a	0.74	0.80	1.08	0.94	0.90	0.96	N
tgifb b	0.73	0.64	0.87	1.11	0.73	0.66	N
tlr3	2.06	1.75	0.85	1.28	1.55	1.21	Y
tlr7	1.71	1.76	1.03	0.85	1.77	2.09	Y
traf5a-1	1.75	1.80	0.05	0.23	0.42	0.19	N
helz2	4.21	9.63	2.29	0.90	1.60	1.77	Y

Bold font identifies genes of interest used in qPCR.
Highlighted cells identify instances with > 2-fold change difference.

Table 2-4 Functions of the genes of interest examined in this study.

Gene	Function
Pattern Recognition Receptors (PRRs)	
tlr3	Recognize molecular patterns specific to microorganisms;
tlr7	Recognize uridine-containing single-stranded RNAs of viral origin or guanosine analogs
dhx58 (alias *lgp2*)	Regulator of DDX58/RIG-1 and IFIH1/MDA5
ifna	Has potent antiviral, antiproliferative and immunomodulatory properties
ifng	Activator of macrophages, and potentiate the antiviral effects of the type I interferons
Signal Transduction	
stat1a and c	Signal transducer and transcription activator that mediates cellular responses to interferons, cytokines and other growth factors
irf1a	Regulates transcription of interferon and interferon-inducible genes, host response to viral and bacterial infections, regulation of many genes expressed during inflammation and immune responses
irf7b	Regulates the transcription of type I interferon genes and interferon-stimulated genes
Immune Effectors	
isg15a	Modulates protein function by ISGylation
mxb	Antiviral activity against wide range of RNA viruses and some DNA viruses
rsad2a and b (alias *viperin*)	Inhibits wide range of DNA and RNA viruses
helz2	Acts as a transcriptional coactivator for a number of nuclear receptors
gig1a	Promotes cell proliferation, chemotaxis, angiogenesis and cell adhesion
5loxb	Biosynthesis of eicosanoids
pgds	Catalyzes the conversion of PGH2 to PGD2

Functions taken from https://www.genecards.org/ on July 19, 2021

Table 2-5. qPCR primers selected for gene expression analysis, showing amplicon size and amplification efficiency for each set of pooled cDNA (i.e, Farm.NA, Wild and Farm.EO), as well as average amplification efficiency values used for relative quantity (RQ) calculations. qPCR cycling conditions were the same for every primer pair (as described in Section 2.5).

Gene Name (GenBank Accession Number)	Nucleotide Sequence (5'-3')	Amplicon Size (bp)	Farm.NA Eff. (%)	Wild Eff. (%)	Farm.EO Eff. (%)	Average Efficiency (%)
Toll-like receptor 3 (*tlr3*) (AKE14222)	F: AATATGGCGCTGGTGAAGAG R: CGCAAAGGTGAACACTGAGA	135	99.6	100.0	95.9	98.5
Toll-like receptor 7 (*tlr7*) (CCX35457)	F: CACCAACACAGAGCTGGAGA R: GCCTTGGAAAACTTGCTGAG	184	102.5	101.7	104.8	103.0
RNA helicase lgp2 (*lgp2*, alias *dhx58*) (NP_001133649)	F: TCCAAGACCCGTAAAAGCAC R: GGTGGAGATCAGGAGGTTGA	189	94.5	90.2	98.2	94.3
Interferon alpha (*ifna*) (NP_001117182)	F: TCCGACACCACTACGGTCA R: CCTCAACCTCGGCATCAT	138	93.9	88.6	88.6	90.4
Interferon gamma (*ifng*) (NM_001171804)	F: CCGTACACCGATTGAGGACT R: GCGGCATTACTCCATCCTAA	133	96.5	97.8	97.3	97.2
Signal transducer and activator of transcription 1 (*stat1a*) (BT045567)	F: GACTGGGAAAATGTGGCTGT R: CATGTGAACAGGGTCCTCCT	180	93.7	92.0	93.6	93.1
Signal transducer and activator of transcription 1 (*stat1c*) (DW551983)	F: GGTCCACACAAATCAACGTG R: CTTTGCAGGGCCTTCTTCTT	154	99.1	97.8	99.4	98.7
Interferon regulatory factor 1 (*irf1a*) (NM_001123645)	F: GCAATGAAGTAGGCACAGCA R: CGCAGCTCTATTTCCGTTTC	100	94.7	93.9	95.6	94.7
Interferon regulatory factor 7 (*irf7b*) (NM_001171850)	F: GTCAGTGGTAAAATCAACACGC R: CACCATCATGAAACGCTTGGT	105	94.3	92.8	94.4	93.8
Interferon stimulated gene 15a (*isg15a*) (BT049918)	F: AAAGTGGCCACAACAAAGCAG R: ATAGGAGCGGGCTCCGTAATC	140	92.2	95.7	92.4	93.4
Interferon-induced GTP-binding protein MX paralogue b (*mxb*) (NM_001139918)	F: ACGCACCACTCTGGAGAAAT R: CTTCCATTTCCCGAACTCTG	184	82.9	94.3	98.4	91.9
Radical S-adenosyl methionine domain containing protein 2 paralogue a (*rsad2a,* alias *viperin* paralogue a) (NM_001140939)	F: ACCATTTTACCCGACAGTGC R: TCCCCAAGAAATCACCTCTG	183	101.1	99.7	100.4	100.4
Radical S-adenosyl methionine domain containing protein 2 paralogue b (*rsad2b,* alias *viperin* paralogue b) (DY728694)	F: TTCCTGGCATGGATAGGTGT R: CTTGGAGTTGTCGCTGGTTT	113	100.2	100.1	107.6	102.6
Helicase with zinc finger domain 2 a (*helz2*, alias *pric285a*) (XM_014168093.1)	F: GCAAGGTTGGGTATGAGGAA R: TTCGGAGTTGCTCCAGTCTT	149	102.46	98.97	100.55	100.66
Gig1 protein (*gig1a*) (BT044028)	F: GTTCTGGGTTTGGTCGTCAC R: CTGTTCTGGAAGGGATGGAA	151	86.5	83.9	84.6	85.0
Arachidonate 5-lipoxygenase b (*5loxb*) (DW555519)	F: ACTGCTGTGGGTTTCCCAAG R: GACAGCAGCGTGATGTGCAG	98	102.6	95.4	104.8	100.9
Prostaglandin-D synthase (*pgds*) (BT125535)	F: GGTGCTCAACAAGCTCTACA R: GCAGGAAAGCGATGTTGTCA	114	92.5	85.8	91.2	89.8

Table 2-5 Continued.

Gene Name (GenBank Accession Number)	Nucleotide Sequence (5'-3')	Amplicon Size (bp)	Farm.NA Eff. (%)	Wild Eff. (%)	Farm.EO Eff. (%)	Average Efficiency (%)
Eukaryotic translation initiation factor 3 subunit D (*eif3d*) (GE777139)[a]	F: CTCCTCCTCCTCGTCCTCTT R: GACCCCAACAAGCAAGTGAT	105	106.3	98.1	108.1	104.2
60S ribosomal protein 32 (*rpl32*) (BT043656)[a]	F: AGGCGGTTTAAGGGTCAGAT R: TCGAGCTCCTTGATGTTGTG	119	102.2	99.1	99.2	100.2

Only *Salmo salar* (taxid:8030) sequences were used for primer identification.
[a]Normalizers, which are also bolded.
[b]Amplification efficiencies were calculated using a 5-point 1:3 dilution series starting with cDNA representing 10 ng of total input RNA. See Materials and Methods for details.
All primers were from Caballero-Solares et al., 2017

In the current study, we explored the transcript expression stability of several candidate normalizer (also known as endogenous control, housekeeping, or reference genes) genes (i.e., *eif3d, rpl32, ef1a, abcf2,* and *polr*2), using 50% of the individuals included in the actual qPCR study. The CT values were measured for each of those genes using diluted cDNA representing 5 ng of input total RNA and analyzed using geNorm in qbase+ software [Biogazelle, Gent, Belgium (Vandesompele et al., 2002)]. Both eukaryotic translation initiation factor 3 subunit D (*eif3d*) and 60S ribosomal protein L32 (*rpl32*) were recommended by geNorm, with M-values 0.415 and 0.421, respectively (Figure 2-5 and Table 2-6). For further confirmation that candidate normalizer genes had stable transcript expression across the experiment (i.e. in all cross types and conditions tested), we performed a qPCR experiment (similar to the actual experiment; using 100% of individuals) for some of the candidate normalizers (i.e., *eif3d*, *rpl32*, *ef1a*, and *polr2*) using two 384 plates (3 technical replicates for each sample); the individuals from each group were equally distributed across the 2 plates, such that each plate had at least one representative individual of each group (i.e., time points, injections, and cross types), as well as a linker sample (in triplicate) on each plate. Collectively, it was determined that both *eif3d* and *rpl32* were the most suitable normalizers for the current study based on having the lowest geNorm M-values, which represent an average expression stability (Figure 2-5), relatively low CT range across all individuals, and low average CT differences between groups (Table 2-6), focusing on the most relevant comparisons (i.e., comparisons within the same time point, comparisons between different injections within the same time point, comparisons across cross types).

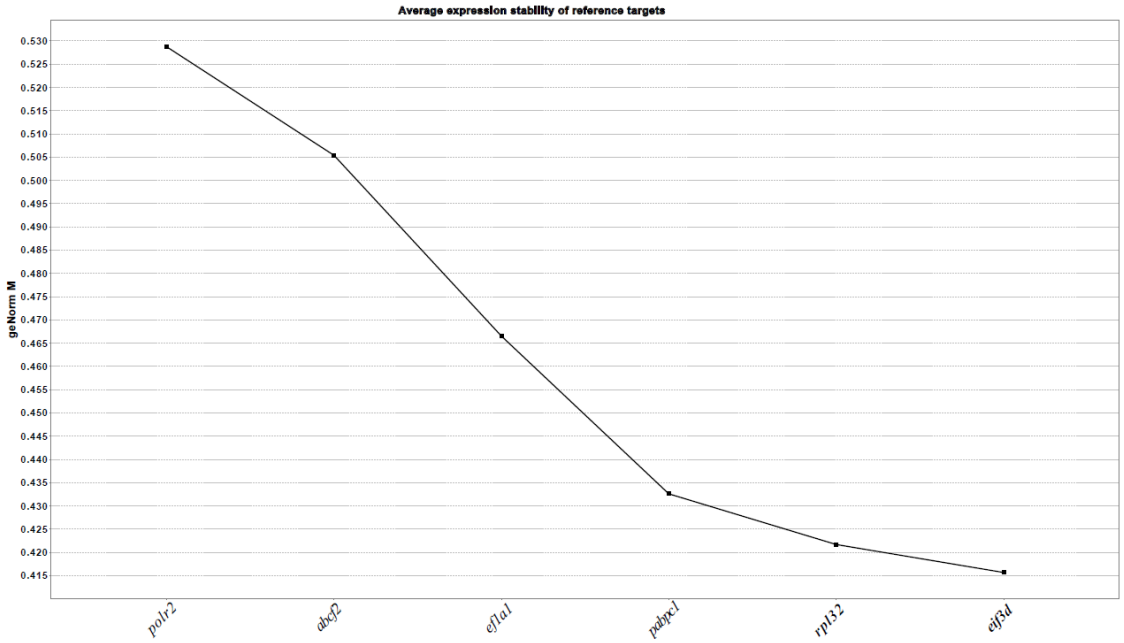

Figure 2-5. The exported geNorm report and M-Value (average expression stability) for all the tested reference genes based on 50% of the individuals from this qPCR study.

Table 2-6. Minimum, maximum and difference of CT values, comparing stability of candidate normalizer genes across stimulation groups tested on all samples for optimal normalizer gene selection for use in the experiment.

	Treatment	Minimum CT	Maximum CT	Difference
eif3d	T_0	21.6	22.1	0.5
	PBS 24 hpi	21.6	22.1	0.5
	pIC 24 hpi	21.8	22.2	0.4
	PBS 72 hpi	21.7	22.2	0.5
	pIC 72 hpi	21.5	22.1	0.6
	Overall	21.5	22.2	0.7
rpl32	T_0	19.1	19.6	0.5
	PBS 24 hpi	18.9	19.6	0.7
	pIC 24 hpi	19.1	19.7	0.6
	PBS 72 hpi	18.8	19.2	0.4
	pIC 72 hpi	18.9	19.4	0.5
	Overall	18.8	19.7	0.9
ef1a1	T_0	19.9	20.1	0.2
	PBS 24 hpi	20.0	20.7	0.7
	pIC 24 hpi	20.4	20.9	0.5
	PBS 72 hpi	19.7	20.1	0.4
	pIC 72 hpi	19.8	20.3	0.5
	Overall	19.7	20.9	1.2
polr2	T_0	24.4	24.8	0.4
	PBS 24 hpi	25.0	25.5	0.5
	pIC 24 hpi	24.9	25.4	0.5
	PBS 72 hpi	24.7	25.0	0.3
	pIC 72 hpi	24.1	24.4	0.3
	Overall	24.1	25.5	1.4

T_0 = time zero sampled fish, no injections.
PBS 24 hpi = PBS injected, sampled 24 hours post-injection.
PBS 72 hpi = PBS injected, sampled 72 hours post-injection.
pIC 24 hpi = pIC injected, sampled 24 hours post-injection.
pIC 72 hpi = pIC injected, sampled 72 hours post-injection.

After primer quality testing was completed and normalizer genes (*rpl32* and *eif3d*) were selected, the relative quantity (RQ) of the transcript levels of the selected GOIs were analyzed using qPCR in technical triplicates. This was done for eight biological replicates per each of the six cross types for a total of 240 samples. For each transcript, two 384-well plates were run, an NTC, containing all reaction components except for cDNA, was run on each plate. A linker sample was also run on each plate to check inter-plate variability. For each GOI, the sample with the lowest expression (after normalization) was set as the calibrator sample (relative quantity [RQ]=1.0).

2.6 Statistical Analyses

The expression of each gene of interest (GOI) was normalized to the expression of *rpl32* and *eif3d*. RQ of each qPCR target of each individual relative to a calibrator sample (i.e. sample with the lowest normalized expression within each gene), was calculated using CT values, and the amplification efficiency of each primer pair, collected from ViiA7 Software. These values were then used in a calculation using a qBase relative quantification framework in Excel (Hellemans et al., 2007). A Grubb's test was used to determine if any sample was considered a significant outlier. If a sample was deemed an outlier by the test in seven or more of the 17 total genes analyzed (Table 2-7), then it was removed from the study; a total of four samples were omitted from this study: two Hyb.EO $_{W♀}$ samples and two Hyb.EO $_{F♀}$ samples (Table 2-7). Assumptions of normality were checked using a Kolmogorov-Smirnov test. I first compared differences in gene expression at t_0 among cross types using one-way ANOVA. This allowed me to assess if there were differences in basal gene expression among the cross types. Next, I evaluated differences in the innate immune response (gene expression) associated with treatment

and cross type. First, I used redundancy analysis (RDA) with all data using the R package *vegan* (Oksanen et al. 2017) with cross type and treatment as constraining factors to explain variation in gene expression. An RDA is an extension of a principal component analysis

Table 2-7. Grubb's test results showing the sample name, and the number of genes for which that sample was an identified outlier. Cross type abbreviations: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), Wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with Wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with Wild male (Hyb.NA $_{F♀}$).

Sample Name	Fish Number	Number of Genes Outlier was Identified
Wild PBS 24 hpi	6	2
Wild PBS 72 hpi	5	2
Wild pIC 72 hpi	2	2
Farm.NA t_0	5	3
Farm.NA PBS 24 hpi	4	2
Farm.NA PBS 72 hpi	4	4
Farm.NA pIC 72 hpi	5	2
Hyb.EO$_W$♀ t_0	1	10
Hyb.EO$_W$♀ t_0	7	4
Hyb.EO$_W$♀ PBS 24 hpi	6	5
Hyb.EO$_W$♀ PBS 72 hpi	2	7
Hyb.EO$_F$♀ t_0	1	9
Hyb.EO$_F$♀ PBS 72 hpi	3	10

Bold font identifies where 1 outlier fish was removed from statistical analysis

(PCA), except RDA models response variables as a function of explanatory variables (Zuur et al., 2007). Therefore, this multivariate analysis provides the opportunity to incorporate all the data into a single analysis, and allows a simple visualization of how the various treatments and cross types differ from one another. Gene loadings on RDA axes help determine which genes contribute most (i.e., magnitude) and in what way (i.e. direction) to the differences detected. I used an ANOVA-like permutation test in *vegan* (anova.cca function) to determine if constraining factor(s) were significant in the RDA model. To further investigate differences in genes expression, a two-way ANOVA with treatment and cross type as presumed fixed factors was carried out for each time point (24 and 72 hpi) to compare pIC vs PBS treatments. If the effect of one or both factors was found to be statistically significant, a Tukey's post-hoc test was performed to compare groups. All the above analyses were done using RStudio (version 1.2.5019).

3. Results

3.1 Cross Type Differences in Basal Expression of *pgds*

Of all 17 genes tested, only one gene, *pgds* (Figure 3-1), showed significant differences ($p < 0.05$) using ANOVA, in basal (i.e., constitutive) head kidney transcript expression among cross types. In the Farm.NA hybrid cross (Hyb.NA $_{F♀}$), *pgds* transcript expression was significantly higher than in both the Wild and one of the Farm.EO hybrid (Hyb.EO $_{W♀}$) crosses, but did not differ from all other crosses. This gene also showed a similar pattern to several other genes: *tlr3, dhx58, stat1c, irf1a, irf7b, rsad2b, isg15a, stat1a, mxb, gig1a,* and *rsad2a* (Figure 3-2 A-J, O), in that Farm.NA and Hyb.NA $_{F♀}$ show the highest expressions when compared to the other pure and hybrid crosses, respectively, as discussed below.

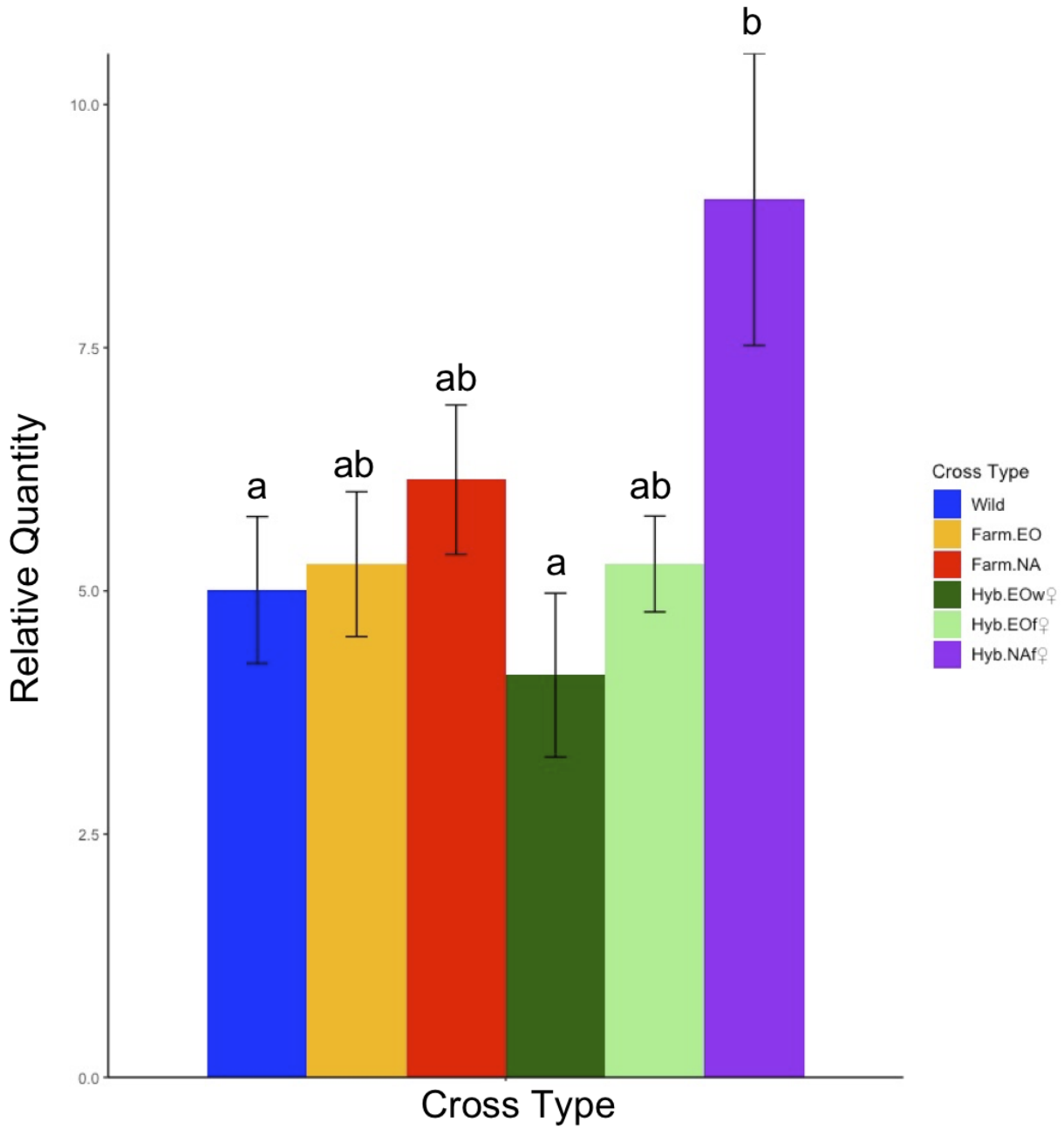

Figure 3-1. Bar graph of relative quantities (mean ± SE) of *pgds* transcript among Atlantic salmon cross types that showed statistically significant ($p < 0.05$) differential basal transcript expression between cross types at t_0. Cross type abbreviations in legend: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with wild male (Hyb.NA $_{F♀}$).

3.2 Basal Expression of Transcripts that Are Non-Significant for Cross Type

For 16 of the 17 GOI transcripts tested, there were no significant differences ($p < 0.05$) in expression at t_0 between cross types (Figure 3-2). However, genes *tlr3*, *dhx58*, *stat1c*, *irf1a*, *irf7b*, *rsad2b*, *isg15a*, *stat1a*, *mxb*, *gig1a*, *helz2* and *rsad2a* all showed a similar non-significant trend of Farm.NA having higher expression than any other pure cross and the related hybrid, Hyb.NA $_{F♀}$, having higher expression than any other hybrid cross (Figure 3-2 A-K, O). The genes in which the Wild cross has the lowest expression (although not significantly lower than any other cross) when comparing the three pure crosses include *tlr3*, *stat1c*, *irf1a*, *rsad2b*, *isg15a, ifng,* and *5loxb*. Moreover, *rsad2b* and *isg15a* show that Wild has the lowest expression overall (again, not significantly lower than any other cross). The genes *mxb*, *gig1a* and *tlr7,* (Figure 3-2 I, J, L) showed a different non-significant trend with Farm.EO having the lowest expression of the pure crosses, contrasting with the previous pattern (Figure 3-2 A, C, D, F, G) where the Wild cross generally showed the lowest expression. The pattern for *ifna* showed an opposite non-significant trend between the pure crosses and the hybrid crosses, such that Wild had the highest expression of the pure crosses, but Hyb.NA $_{F♀}$ had the highest expression of the hybrid crosses (Figure 3-2 M). There is a similar trend for *tlr7* and *ifna* (i.e., highest expression in Hyb.NA $_{F♀}$), although not significantly different from any other cross type (Figure 3-2 L-M). For *ifng* and *5loxb* Farm.EO had the highest expression of the pure crosses (although not significantly different from any other cross type) (Figure 3-2 N and P).

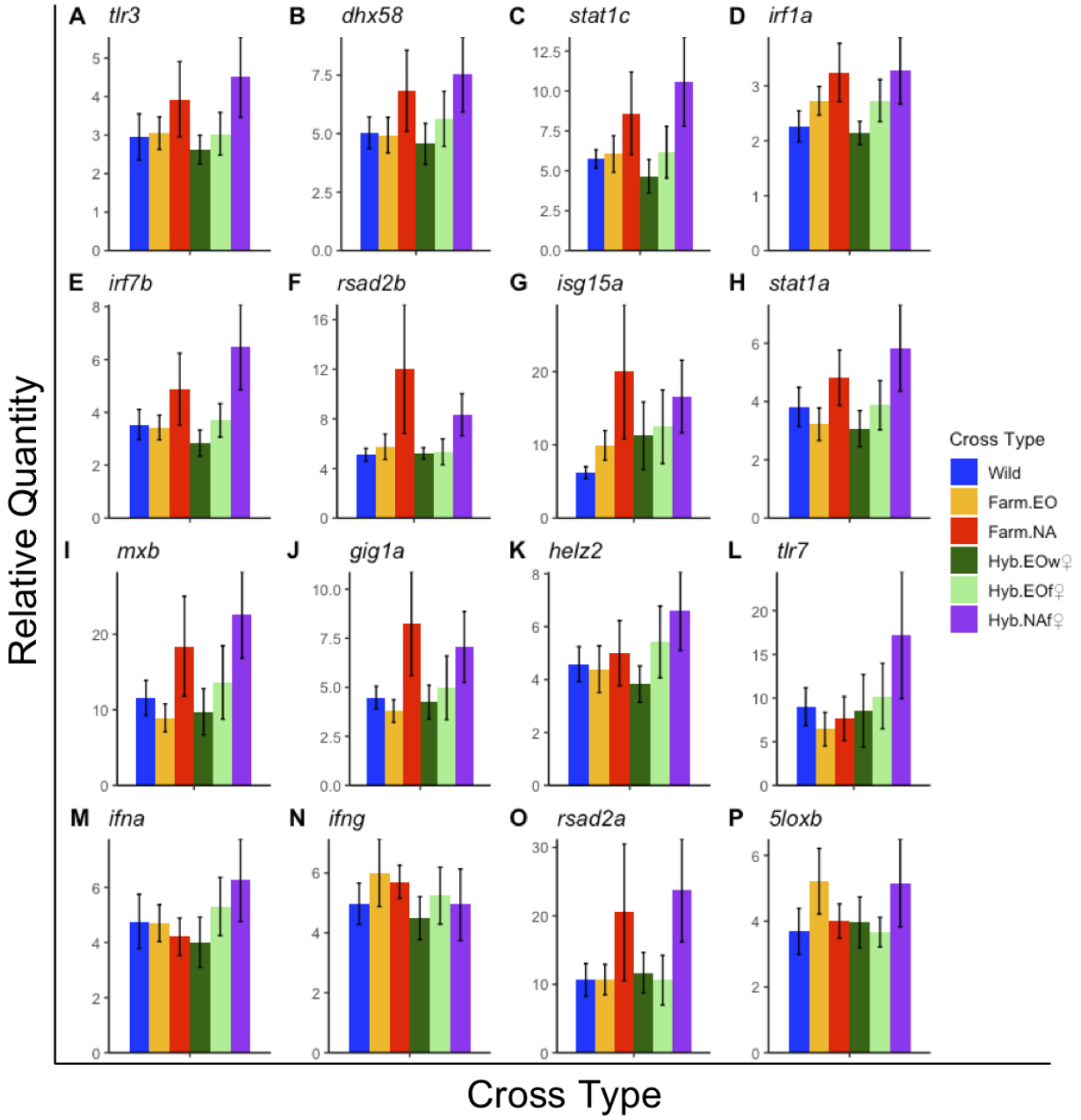

Figure 3-2. Bar graphs of relative quantities (mean ± SE) of antiviral gene expression among Atlantic salmon cross types that showed statistically non-significant ($p > 0.05$) differences based on ANOVA between cross types at t_0. Cross type abbreviations in legend: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), Wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with Wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with Wild male (Hyb.NA $_{F♀}$).

3.3 Multivariate Analysis

An RDA that included all samples, grouped the control (PBS 24 hpi and PBS 72 hpi) and t_0 samples together (Figure 3-3). In addition, the RDA separated the pIC 24 and pIC 72 hpi samples from each other on the second RDA axis as well as from both the control and t_0 samples along the first RDA axis. These differences along the first RDA axis were driven by the majority of the genes which were upregulated following pIC injection (i.e. having positive loadings), with the exception *5loxb* and *pgds* which were downregulated (negative loading; see Figure 3-3). The RDA model showed that both cross type and treatment were significant factors in the model (p-value = 0.030 and 0.001, respectively). I also performed an RDA on both pIC groups vs both PBS groups without t_0 (Figure 3-4). This RDA model showed that treatment was a significant factor (p-value = 0.001), but cross type was not a significant factor (p-value = 0.063). When t_0 was included in the full RDA versus the RDA without t_0, the results for contributing genes were qualitatively similar. Given the significance of treatment and cross type, I next performed two-way ANOVAs on each GOI to determine any cross type differences.

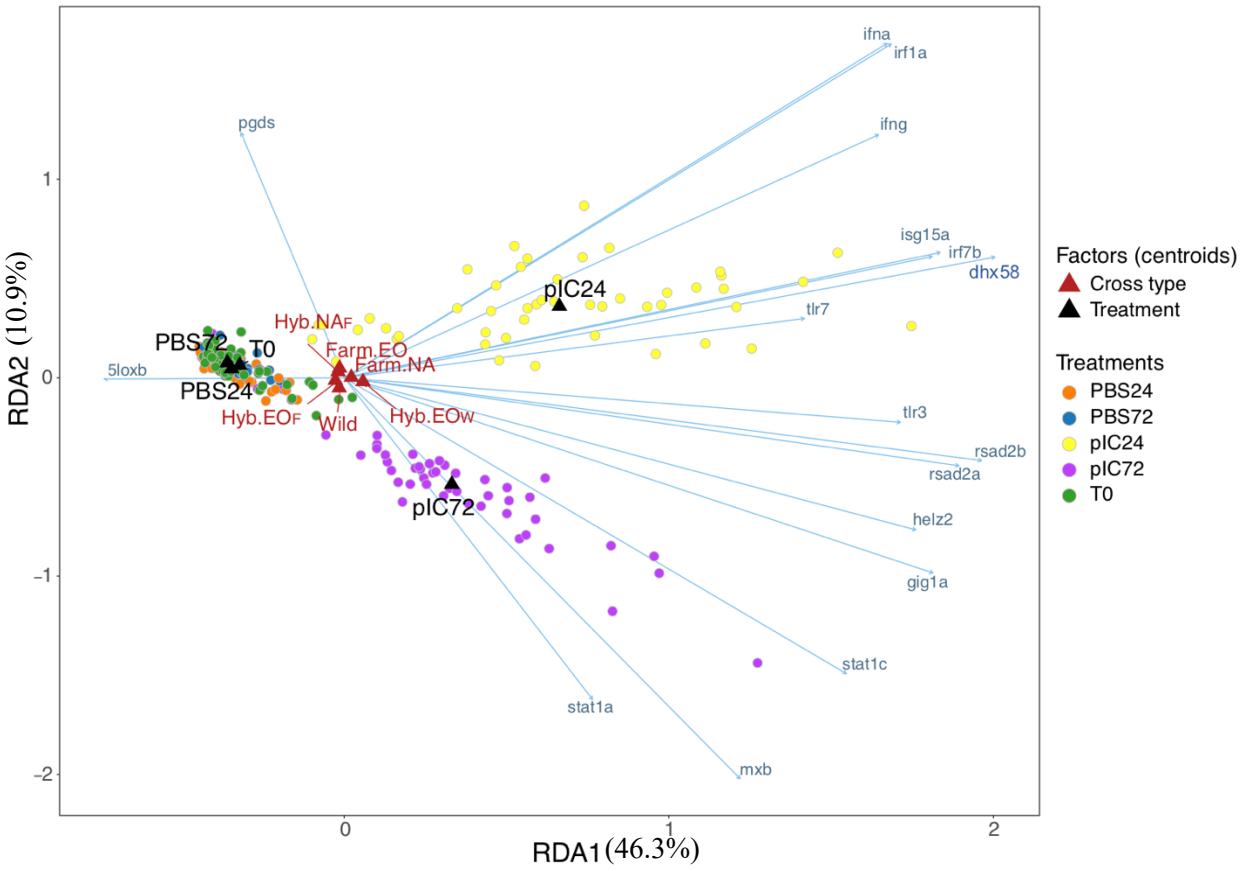

Figure 3-3. Redundancy analysis (RDA) of all treatment groups showing the first and second axes. Cross type abbreviations in figure: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), Wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with Wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with Wild male (Hyb.NA $_{F♀}$). Percent variance explained for each RDA axis shown in brackets.

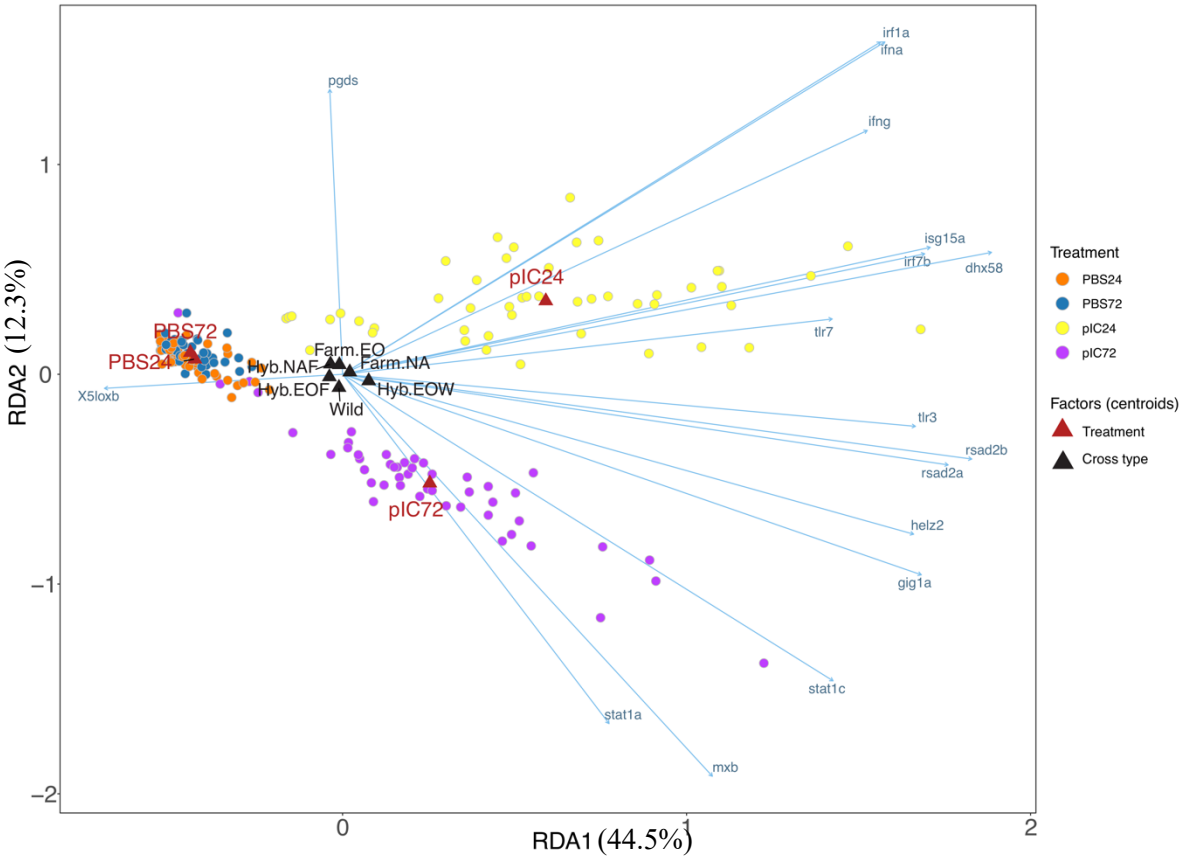

Figure 3-4. Redundancy analysis (RDA) of all PBS and pIC treatment groups excluding t_0 showing the first and second axes. Cross type abbreviations in figure: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), Wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with Wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with Wild male (Hyb.NA $_{F♀}$). Percent variance explained for each RDA axis shown in brackets.

3.4 Cross Type Differences of Target Transcript Expression in Response to pIC Injection

Significant differences ($p < 0.05$) in the response to pIC injection among cross types were detected only at 24 hpi and for only four of the genes: *ifna*, *isg15a*, *rsad2a*, and *rsad2b* (Figure 3-5), which are all interferon pathway genes. In the pIC-stimulated fish 24 hpi, *ifna* transcript expression was highest in Farm.EO and lowest in Wild, with Farm.EO differing significantly from Wild, Hyb.EO $_{F♀}$, and Hyb.NA $_{F♀}$ (Figure 3-5 A). Furthermore, for the pIC 24 hpi treatment, Farm.NA had significantly higher *ifna* transcript expression than in the Wild and Hyb.NA $_{F♀}$ groups (Figure 3-5 A). Transcript expression of *isg15a* (Figure 3-5 B) for pIC-stimulated fish at 24 hpi was highest in Farm.EO and Hyb.EO $_{W♀}$, and lowest in Wild, with Wild and Hyb.NA $_{F♀}$ differing significantly from Farm.EO and Hyb.EO $_{W♀}$. Similarly, transcript expression of *rsad2a* for pIC-stimulated fish at 24 hpi was highest in Hyb.EO $_{W♀}$ and Farm.EO, which differed significantly from Wild and Hyb.NA $_{F♀}$ (Figure 3-5 C). Lastly, in the pIC-stimulated fish 24 hpi, *rsad2b* transcript expression was also highest in Hyb.EO $_{W♀}$ and lowest in Wild, similar to *rsad2a* and *isg15a*, with the two differing significantly (Figure 3-5 D).

The overall expression profiles of *rsad2a* and *rsad2b* (Figure 3-5 C-D) show that they are significantly and similarly upregulated in pIC compared with PBS groups at both 24 and 72 hpi time points. The overall expression profile of *isg15a* (Figure 3-5 B) shows that it is significantly upregulated by pIC (compared with time-matched PBS controls) at both time points, but more strongly induced in pIC 24 hpi compared with pIC 72 hpi.

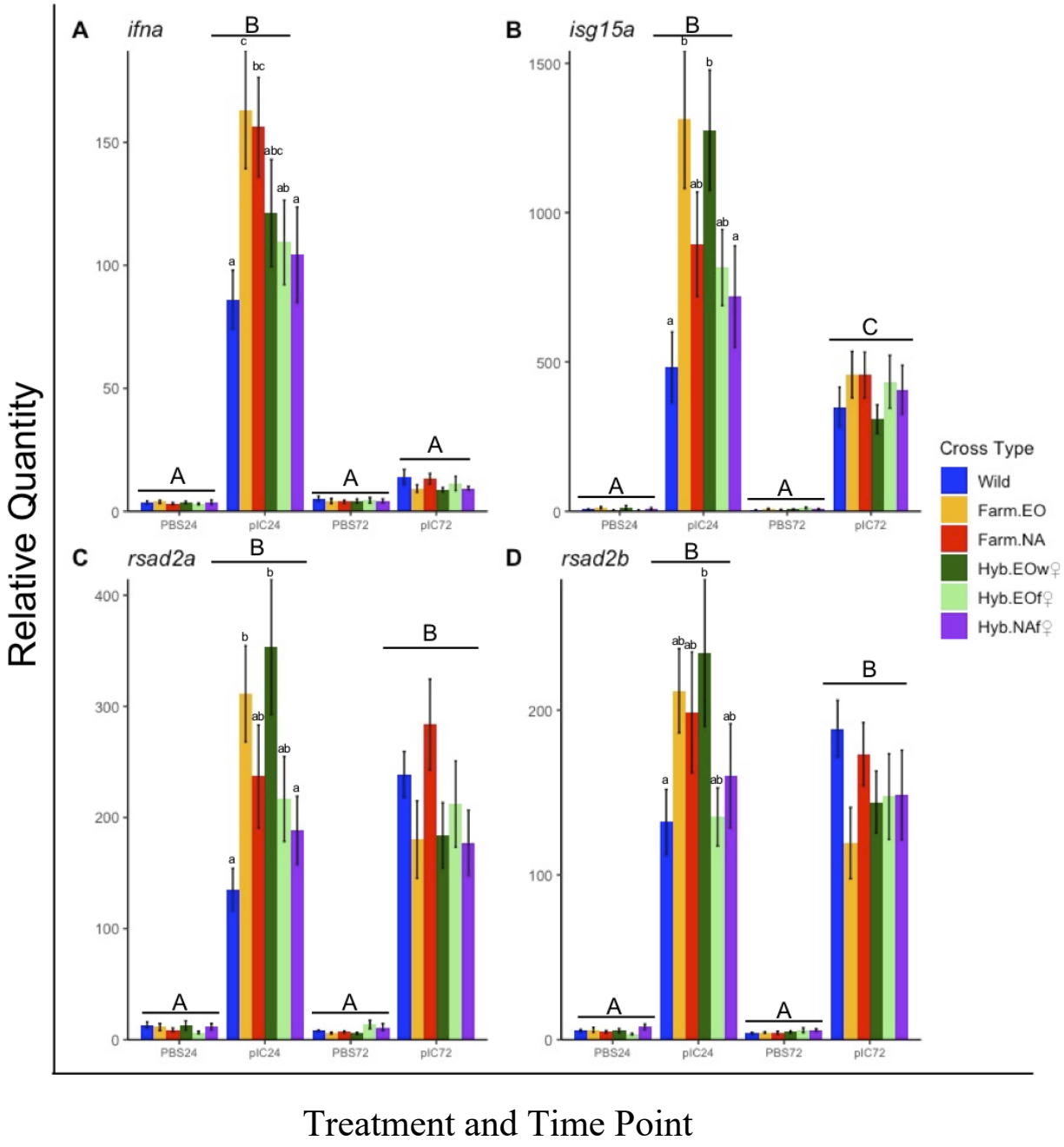

Figure 3-5. Effect of pIC versus PBS (control) intraperitoneal injection on head kidney expression of genes that were influenced by cross type. Mean ± SE relative quantities shown, with significant induction across all time points indicated by uppercase letters (two-way ANOVA, Tukey's post-hoc test, $p < 0.05$), and between cross types in a given time point by lowercase letters (two-way ANOVA, Tukey's post-hoc test, $p < 0.05$). Cross type abbreviations in legend: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), Wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with Wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with Wild male (Hyb.NA $_{F♀}$).

3.5 Target Transcript Expression in Response to pIC Injection that Were Non-Significant for Cross Type

A two-way ANOVA showed that 15 genes were found to be significantly upregulated by pIC (compared with time-matched PBS-injected controls) for at least one time point post-injection (Figures 3-5 and 3-6). The expression of *tlr3*, *gig1a* and *helz2* (Figure 3-6 A-C), like that of *rsad2a* and *rsad2b* (Figure 3-5 C-D), showed significant and comparable upregulation by pIC compared with PBS groups at both 24 and 72 hpi time points. The expression of *tlr7*, *dhx58*, *ifng* and *irf7b* (Figure 3-6 D-G), like that of *isg15a* (Figure 3-5 B), also showed significant upregulation by pIC (compared with time-matched PBS controls) at both time points, but expression was significantly higher in pIC 24 hpi than pIC 72 hpi fish.

Similarly, the expressions of *stat1c* and *mxb* (Figure 3-6 H-I) were also significantly upregulated by pIC (compared with time-matched PBS controls) at both time points, but with expression higher in pIC 72 hpi than in pIC 24 hpi fish. The expression of *stat1a* (Figure 3-6 J) was significantly upregulated by pIC (compared with time-matched PBS controls) only at 72 hpi. Contrastingly, the expression of *irf1a* (Figure 3-6 K), like that of *ifna* (Figure 3-5 A), was significantly upregulated by pIC (compared with time-matched PBS controls) only at 24 hpi. The expression of *5loxb* (Figure 3-6 L) was significantly and comparably downregulated in pIC compared with PBS groups at both 24 hpi and 72 hpi time points. Similarly, *pgds* (Figure 3-6 M) was also significantly downregulated in pIC compared with PBS groups, however, only at the 72 hpi time point.

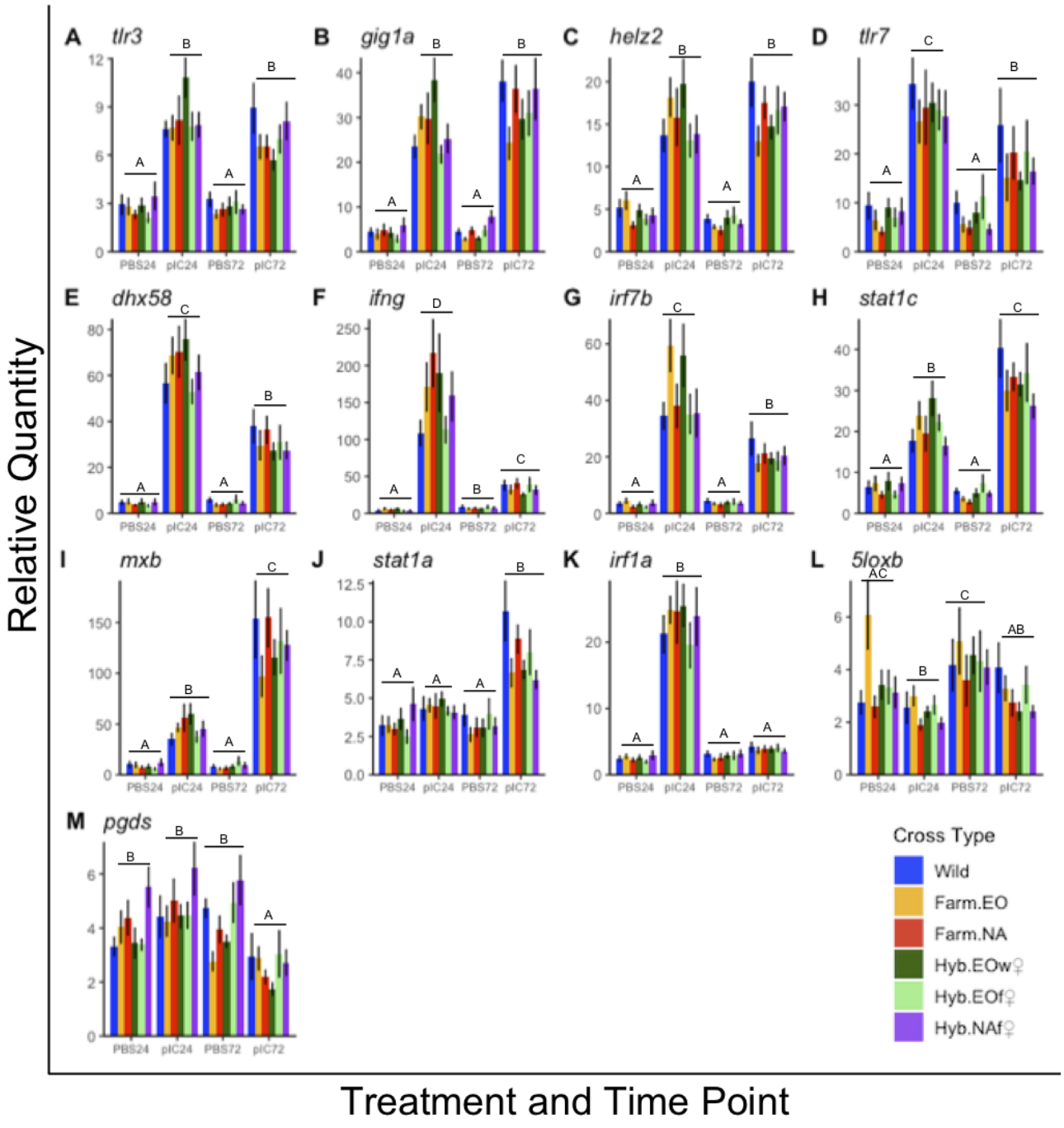

Figure 3-6. Effect of pIC versus PBS (control) intraperitoneal injection on head kidney expression of genes that did not show significant differences among cross types at either time point. Mean ± SE relative quantities shown with significant induction indicated by uppercase letters (two-way ANOVA, Tukey's posthoc test, $p < 0.05$). Cross type abbreviations in legend: pure wild (Wild), pure European farmed (Farm.EO), pure North American farmed (Farm.NA), Wild female crossed with Farm.EO male (Hyb.EO $_{W♀}$), Farm.EO female crossed with Wild male (Hyb.EO $_{F♀}$), and Farm.NA female crossed with Wild male (Hyb.NA $_{F♀}$).

4. Discussion

Aquaculture is a fast-growing food sector and is economically important worldwide. Salmon aquaculture has the risk of escaped farmed animals that can potentially interbreed with the conspecific wild populations causing the alteration of fitness traits that can lead to demographic decline. My study investigated the effect of hybridization on innate immune response in Atlantic salmon by comparing pure Wild and farmed cross types as well as related hybrid cross types. The differences in the basal (i.e. constitutive) expression and the innate antiviral-like immune response among the different cross types were examined in this study. Post-injection samples with pIC (compared with time-matched PBS injected controls) were studied at two post-injection time points (24 h and 72 hpi) to assess the innate antiviral-like immune response. The results showed that only one gene, *pgds*, of the 17 targeted genes in this study showed significant differences among cross types prior to injection, indicating some basal differences in gene expression among cross types. I found that PBS injected controls grouped together with the t_0 samples showing they do not significantly differ from pre-injection sampling indicating an overall lack of injection induced response. However, pIC-injected fish grouped separately from each other and from PBS and t_0 fish, and this was driven by the upregulation of 15 of the 17 GOIs (all except *pgds* and *5loxb*) following pIC injection. When induced by pIC injection (compared with time-matched PBS injected controls, four of 17 targeted genes (*ifna, isg15a, rsad2a* and *rsad2b*),) showed significant differences among cross types at 24 hpi.

4.1 Cross Type Differences in Basal Expression of *pgds*

The basal expression (t_0) of the immune relevant genes studied showed that there was a significant cross type difference for *pgds*. The *pgds* transcript levels were significantly lower in the Wild and Hyb.EO $_{W♀}$ cross types compared with Hyb.NA $_{F♀}$ (Figure 3-1). Eicosanoids involved in immune regulatory activity are found at the site of inflammation and can modulate the inflammatory response in fish (Rowley et al., 1995) and it is known that *pgds* helps to mediate the inflammatory response (Gómez-Abellán and Sepulcre, 2016). For example, in uninfected skin from the common carp (*Cyprinus carpio* L.), the most abundant immune-related gene was *pgds*, showing that this is an important transcript, even before infection (Gonzalez et al., 2007). As well, in the gilthead seabream (*Sparus aurata* L.), the presence and level of *pgds* was found to play a role against inflammation caused by bacterial infection, while there was little expression in the control samples (Gómez-Abellán et al., 2015).

The differences in basal (i.e. constitutive) head kidney *pgds* transcript levels among cross types in our t_0 samples resulted in Hyb.NA $_{F♀}$ showing the highest *pgds* expression of the three hybrid cross types (significantly higher than Hyb.EO $_{W♀}$). Also, Hyb.NA $_{F♀}$ had the lowest expression of three of the four antiviral biomarkers that were influenced by cross type in pIC stimulated fish at the 24 hpi time point (with significantly lower expression of *isg15a* and *rsad2a* compared with Hyb.EO $_{W♀}$). This could suggest that the cross type with the highest *pgds* expression (Hyb.NA $_{F♀}$) may have an increased pro-inflammatory response before any injection, which could influence the response to pIC injection in this cross type by decreasing the innate immune response related to viral infection (Heidari et al., 2015). A stronger response to pIC injection in Hyb.NA $_{F♀}$, compared to all other crosses, could be due to the fact that farmed salmon are more resistant to viral infections than wild ones (Gjøen and Bentsen, 1997).

However, disease resistance has been shown in Norwegian farmed salmon, but not to my knowledge in North American farmed salmon yet. The stronger expression of *pgds* could also mean that Hyb.NA $_{F♀}$ were experiencing stress or a previous infection before experimentation.

4.2 Cross Type Differences in *ifna* Expression Response to pIC Injection

When comparing cross types after pIC injection, there were four different genes that showed significant cross type differences in expression: *ifna*, *isg15a*, *rsad2a* and *rsad2b* (Figure 3-5). The differences in cross type only occurred at 24 hpi, which coincides with the maximum overall upregulation of these genes. For the 24 h pIC stimulated treatment, Farm.EO had significantly higher *ifna* expression than Wild, Hyb.EO $_{F♀}$ and Hyb.NA $_{F♀}$, and Farm.NA had significantly higher *ifna* expression than Wild and Hyb.NA $_{F♀}$ (Figure 3-5A). When a viral infection happens, the IFN response is the first line of defense. Upregulation of IFNs, such as *ifna*, induces the innate immune response related to viral infection and in fish, this is very similar to the mammalian response (Zhang and Gui 2012). It has been shown that the IFN pathway is well conserved between higher vertebrates and salmonids (Robertson, 2008; Skjesol et al., 2010; Levraud et al., 2019). The importance of IFNs in the innate immune response is to induce an antiviral state in infected cells (Robertson, 2018). Some IFNs also have the ability to activate natural killer cells, which would increase the sensitivity of cells to recognize an infection (Schneider et al., 2014). In a cell culture study, *ifna* was induced in response to salmon alphavirus (SAV3), however it was not induced when SAV3 was injected into Atlantic salmon (Chang et al., 2016). In another cell study, using Atlantic salmon head kidney and blood leukocytes, *ifna* successfully inhibited infection by infectious pancreatic necrosis virus (IPNV)

(Svingerud et al., 2012). This study also showed that the ability of *ifna* to induce other antiviral genes such as *mx* and *isg15* peaked at 24 h post-infection (Svingerud et al., 2012).

The two pure farmed crosses (Farm.NA and Farm.EO) had the highest *ifna* expression in response to pIC stimulation at 24 hpi, and showed a 1.8-fold difference from Wild. This could suggest the hypothesis that these farmed cross types would have a higher resistance to viral infections compared with the Wild cross type. This could be caused by a purposeful selection process for disease resistant salmon in aquaculture, and would support this hypothesis which was proposed by Gjøen and Bentsen (1997). The expression of *ifna* in response to pIC was only significantly upregulated only at 24 hpi (and not 72 hpi). This could suggest that Farm.EO and Farm.NA salmon will have a faster and/or stronger activation of their innate antiviral-like immune responses than the other cross types due to the significantly higher expression of *ifna*. The expression of *ifna* also agrees with previous studies done with pIC at various time points after injection, suggesting it is important at the beginning of an infection due to its higher expression at 24 than 72 hpi (Skjesol et al., 2010; Caballero-Solares et al., 2017; Levraud et al., 2019).

4.3 Cross Type Differences in *isg15a* Expression Response to pIC Injection

Another transcript that showed significant cross type differences in expression after pIC injection was *isg15a.* At the 24 hour post-stimulation time point, *isg15a* expression was significantly higher in Farm.EO and Hyb.EO $_{W♀}$ compared with Wild and Hyb.NA $_{F♀}$ (Figure 3-5B). Such interferon stimulated genes (ISGs), like *isg15a, rsad2*, and *mxb*, are activated by the IFN signalling pathway (Poynter and DeWitte-Orr, 2016) and *isg15* modulates protein function by ISGylation (Sadler and Williams, 2008). The function of these ISGs is to limit viral

replication and infectivity (Poynter and DeWitte-Orr, 2016). In both mammals and fishes, the importance of *isg15* has been identified as an early response to viruses (Verrier et al., 2011). Both *isg15* and *rsad2* have been identified among the 72 ancestral (to mammals and fish) ISGs (Levraud et al., 2019). In addition, *isg15* encodes one of the proteins that are induced earliest and most strongly to inhibit viral replication (Sadler and Williams, 2008; Svingerud et al., 2012). After infection with SAV3, *isg15* was overexpressed in the Atlantic salmon macrophage-like T_0 cell line (Xu et al., 2010). The difference found in our study could suggest that Farm.EO and Hyb.EO $_{W♀}$ are more equipped to limit viral replication and infectivity than Wild and Hyb.NA $_{F♀}$ as they had a significantly higher expression for pIC 24 hpi, and also that EO may have a stronger antiviral-like immune response than Wild and NA, as EO is the common parent between these two crosses. This difference could further support the hypothesis that European farmed fish have been selected for disease resistance (Gjøen and Bentsen, 1997). However, the StofnFiskur strain of fish from Benchmark Genetics (i.e. Farm.EO) have not undergone selection for disease resistance. Benchmark Genetics StofnFiskur strain of salmon were established in the early 1980s from Norwegian fish, which would have taken place before Norwegian strains had undergone directed selection for disease resistance in 1993, and have been kept in disease-free containment since (Gjøen and Bentsen, 1997; https://bmkgenetics.com/about/our-salmon-strains/).

4.4 Cross Type Differences in *rsad2a* and *rsad2b* in Response to pIC Injection

Another ISG is radical S-adenosyl methionine (SAM) domain-containing 2 (*rsad2*). Aliases (i.e. synonyms) of this gene include virus inhibitory protein, endoplasmic reticulum (ER)-associated, IFN-inducible (*viperin*) and virus-induced gene 1 (*vig1*) (Eslamloo et al., 2019). An antiviral protein, RSAD2, can be induced by pIC, lipopolysaccharide (LPS), viruses, and

bacteria (Severa et al., 2006; Fitzgerald, 2011). The expression of *rsad2* in mammals is induced through IFN pathways, which may be activated by the detection of viruses or dsRNA through the activation of transcription factors (e.g. IRF3) (Fitzgerald, 2011; Mattijssen and Pruijn, 2012; Helbig and Beard, 2014). Antiviral responsiveness of RSAD2, while greatly studied in mammals has also been observed in teleosts (e.g. Eslamloo et al., 2019) (Rise et al., 2008; Rise et al., 2010). In this study, two paralogues of *rsad2* (*rsad2a* and *rsad2b*) were tested. In the pIC-treated fish at the 24 hour post-stimulation time point, head kidney *rsad2a* transcript expression was significantly higher in Farm.EO and Hyb.EO $_{W♀}$ groups compared with Wild and Hyb.NA $_{F♀}$ groups (Figure 3-5C); for *rsad2b*, expression was significantly higher in the Hyb.EO $_{W♀}$ group compared with the Wild group (Figure 3-5D). Studies have shown that teleost fish *rsad2* is induced in response to pathogenic viruses. For example, when post-smolt Atlantic salmon were infected with salmon alphavirus (SAV3) it was found that *rsad2* showed one of the most highly upregulated protein expressions in pancreatic and heart tissues (Moore et al., 2017). The maximum expression in pancreatic and heart tissues for the SAV3 injected group occurred at 7 days post infection; however, for the SAV3 bath immersion group, while expression was still significantly upregulated it was later (Moore et al., 2017). Among the earliest descriptions of this gene was in rainbow trout (*Oncorhynchus mykiss*) leukocytes (Boudinot et al., 1999). It is known that *rsad2* destroys lipid rafts which prevents the budding and release of viruses (Fitzgerald, 2011). It also inhibits the ability of viruses to exit the host cells (Schneider et al., 2014). Induction of *rsad2* in macrophages was observed in chum salmon (*Oncorhynchus keta*), after stimulation with pIC (DeWitte-Orr et al., 2007) as well as in Atlantic cod (Rise et al., 2008, 2010). The cross type differences in my study suggest that Wild and Hyb.NA $_{F♀}$ (which have the highest expression of both *rsad2* paralogues among the pIC-stimulated 24 hour groups) may

have a weaker response to viral infection than Farm.EO and Hyb.EO $_{W♀}$. This hypothesis should be tested in future research involving viral pathogen exposures in a biocontainment facility.

The observed cross type differences in *rsad2* expression response to pIC could be influenced by the geographical similarities of the cross types. Both Wild and Hyb.NA $_{F♀}$ are North American originating cross types, and both Farm.EO and Hyb.EO$_{W♀}$ grouped together suggesting that the EO component of the hybrid overrides that of the NA component. Therefore, it was not unexpected that they grouped together with their geographical neighbours with regard to the trait of innate antiviral-like immune response, which is also true for all of the genes studied. The expressions of both *rsad2a* and *rsad2b* are very similar (e.g. Farm.EO, Farm.NA, and Hyb.EO$_{W♀}$ had the highest expression in both transcripts), suggesting that there was little divergence of regulation or function in these paralogues, which we would expect due to whole genome duplication. However, a limitation of this study is that it is difficult to separate geographic differences from domestication effects in these comparisons.

To summarise, for three of the four genes that showed cross type differences (i.e. *isg15a*, *rsad2a* and *rsad2b*), Farm.EO and Hyb.EO$_{W♀}$ have the highest expression levels for the pIC 24 hpi treatment. Farm.EO also has the highest or second-highest expression for the same treatment in all four genes. There is also a directional effect in the hybridization of Farm.EO and Wild parents for three of the four genes that showed a significant cross type difference (i.e. *isg15a*, *rsad2a*, and *rsad2b*). Hyb.EO$_{W♀}$ consistently had a higher expression than Hyb.EO$_{F♀}$ at the 24 hpi treatment, implying an important effect of the direction of hybridization on the innate immune response. To my knowledge, there have been no studies examining directional effects of hybrid cross type differences, specifically with regards to immune response. However, this result could mean that hybrids who have a European farmed mother would have a stronger immune

response than hybrids who have a European farmed father. Further studies should be done focusing on the direction of hybridization to infer if this has a significant effect on the immune response for hybrids.

4.5 Target Transcript Expression in Response to pIC Injection that Are Non-Significant for Cross Type

The target genes were chosen to include a representation of various pathways in the Atlantic salmon immune response (Eslamloo et al., 2017), including pattern recognition, cytokines, signal transduction, immune effectors and two eicosanoid synthesis-related transcripts. The first step in the host response to a pathogen or pathogen-like stimulus is pattern recognition, and transcripts encoding three PRRs were included in my study: *tlr3*, *tlr7* and *dhx58*. As a PRR *tlr3* recognizes dsRNA (such as pIC) and induces type I interferon (IFN) production (Akira et al., 2006), inhibiting viral replication (Noppert et al., 2007). There was significant upregulation of all three PRR-encoding transcripts at both 24 and 72 hpi in the pIC-injected fish (compared with time-matched PBS-injected controls) in my study, regardless of cross type. While *tlr3* induction by pIC was comparable at both time points, both *tlr7* and *dhx58* had decreased induction at 72 hpi compared with 24 hpi in pIC-injected fish. Caballero-Solares et al. (2017) also studied the effect of intraperitoneal pIC injection on Atlantic salmon head kidney *tlr3* and *tlr7* transcript expression, showing that both genes were significantly upregulated (4.7 to 5.9 fold for *tlr3* depending on diet, and 6.5 to 9.5 fold for *tlr7* depending on diet) compared with PBS injected controls. Collectively, the current study and that of Caballero-Solares et al. (2017) suggest that both *tlr3* and *tlr7* play important roles in response to dsRNA; however, while the expression of these genes was shown to be influenced by diet (Caballero-Solares et al. 2017), they were not influenced by cross type. It is known that *dhx58* plays a role

as a regulator of other dsRNA and ssRNA detectors in mammals (Yu et al., 2011), however in fish it is poorly understood. A previous study done on Atlantic salmon smolts showed that the upregulation of *dhx58* at 24 hpi supported the importance of the transcript in early innate immune response related to viral infection, and was not influenced by diet (Caballero-Solares et al., 2017). The current study supports this finding as *dhx58* was upregulated at 24 hpi and then the expression decreased at 72 hpi; also *dhx58* was not influenced by cross type.

The PRRs trigger a signaling cascade inducing type I IFN production which leads to the initiation of the innate immune response. My transcript expression study included two IFN encoding genes: *ifna* and *ifng*. Signal transducers are responsible for signalling type I and II IFNs. In the current study, the target genes related to signal transduction/transcription factors (i.e. *stat1a*, *stat1c*, *irf1a*, and *irf7b*), were upregulated in this study in accordance with other studies (Caballero-Solares et al., 2017; Eslamloo et al., 2017; Ignatz et al., 2020). While there were no cross type differences in the signal transducer and activator of transcription 1 (STAT1) family paralogues, there were differences in response timing to pIC-induction in my study, suggesting that both paralogues play important but potentially differing roles in the immune response. One paralogue, *stat1c,* was significantly upregulated by pIC (compared with time-matched PBS controls) at both time points, and had significantly higher expression in pIC 72 hpi than 24 hpi. Comparably, *stat1a* was also significantly upregulated by pIC (compared with time matched PBS controls), however only at 72 hpi. Thus, *stat1c* is likely important at both earlier and later time points, while *stat1a* is likely involved in the later response. Both *irf1a* and *irf7b* were upregulated at 24 hpi and then decreased to levels comparable to t_0, PBS 24 and 72 hpi at pIC 72 hpi, suggesting that interferon regulatory factors (IRFs) are important in early signaling transduction. While Caballero-Solares et al. 2017 only considered pIC 24 hpi (and not 72 hpi),

that study showed differences in *stat1a* and *stat1c* transcript expression responses to pIC and diet (5.1 to 6.4 fold induction for *stat1a* compared with time-matched PBS-injected controls, depending on diet; versus 18 to 25 fold induction for *stat1c* compared with PBS controls, depending on diet).

The expression of interferon stimulated genes (ISGs) and immune effectors is activated by the IFN signalling pathway, including genes such as *isg15a*, *rsad2a*, *rsad2b*, *mxb*, and *gig1a*. One other immune related transcript, *helz2*, was included in this study. This transcript has recently been described as a helicase that acts as a transcriptional coactivator for many nuclear receptors that may also play a sensor role (Fusco et al., 2017; Levraud et al., 2019). It was also identified as an ancestral ISG (between mammals and fishes) with conserved components of antiviral immunity (Levraud et al., 2019). It was upregulated at both 24 and 72 hpi in the current study, and the upregulation agrees with the Caballero-Solares et al. (2017) study.

The pIC-associated downregulation of eicosanoid synthesis genes (*5loxb* and *pgds*), which mediate the inflammatory responses (Rowley et al., 1995), co-occurred with the upregulation of antiviral genes in this study. The downregulation of *5loxb* and *pgds* suggests that there was an inhibition of the production of pro-inflammatory eicosanoids in response to pIC. In accordance with a previous study, this could suggest a response to alleviate the pro-inflammatory stimuli from activated macrophages (Caballero-Solares et al., 2017). Furthermore, the significant downregulation of *5loxb* by pIC (compared with time-matched PBS injected controls) at both 24 hpi and 72 hpi suggests that this gene played an important part of the immune response at both time points. Contrastingly, *pgds* was only significantly downregulated by pIC at 72 hpi (compared with time-matched PBS injected controls), suggesting that it may have played an

important role in the innate immune response related to viral infection only at the later time point.

The lack of significant differences between cross types, for all but four genes (i.e. *ifna*, *isg15a*, *rsad2a* and *rsad2b*), is an interesting result which could mean that, with regard to the influence of pIC (i.e. virus-like stimulus) on eicosanoid synthesis, these cross types may be quite similar. The similarity in patterns across these transcripts could imply that part of the immune response of Atlantic salmon does not differ between the North American cross types and the European cross types. While cross type did not significantly affect expression of the transcripts in Figure 3-6, cross type did significantly influence the pIC-associated expression of four genes that are profoundly important for fish antiviral immune responses (*ifna*, *isg15a*, *rsad2a* and *rsad2b*; Figure 3-5). It is important to point out that these results could be different in the wild/natural environment. Under a controlled environment, many genes do not show significant differences; however, in nature, the results could differ.

4.6 Implications

The negative effects of escaped farmed salmon include both ecological and genetic impacts (Thorstad et al., 2008). One of the principal concerns regarding escaped farmed salmon is the consequent potential for hybridization with wild populations (McGinnity et al., 2003; Glover et al., 2017; Bradbury et al., 2020). Recent studies have demonstrated the potential for genetic, fitness and other changes that can occur with the hybridization of escaped salmon (reviewed in Glover et al., 2017). Our results give insight into the genetic implications, specifically the innate immune response of farmed and wild salmon of differing origins, and consequences of hybridization. The results of my study can lead to the hypothesis that the cross

types with a stronger response could potentially mount stronger innate antiviral-like immune responses to viral pathogens. Therefore, different cross types could be differentially susceptible to viral infections. However, consequences of escapes could also be negative for wild populations because it could mean that wild fish are more susceptible to pathogens that originate within fish farms. This hypothesis could be tested in future research involving viral pathogen exposures (in a biocontainment facility). The results of the pIC injection show that the Wild cross type consistently showed the lowest expression at 24 hpi for many of the upregulated GOIs examined, except for *helz2*, *tlr7*, *dhx58*, and *sta1c*. This could mean that, when compared to the other cross types, Wild salmon may have a more difficult time resisting viral infection.

Farmed fish experience selective pressures including that for disease resistance (Gjedrem and Baranski, 2010). The densities that farmed fish experience can be high and therefore the risk for disease transmission is increased, resulting in outbreaks which give the potential to select for disease resistant individuals by using the survivors of a disease outbreak for broodstock (Yáñez et al., 2014). The results that I found show that the Wild cross type has consistently lower expression of some key antiviral biomarker genes (i.e. *ifna*, *isg15a*, *rsad2a*, and *rsad2b*) in head kidney tissues of pIC exposed salmon at the 24 hpi time point, than both of the farmed cross types. Furthermore, in three of the four genes that showed significant cross type differences in response to pIC at the 24 hpi time point (i.e. *ifna*, *isg15a*, and *rsad2a*), one or both farmed cross types differed significantly from the Wild cross type. This could mean that a selection process for disease resistant fish may have been carried out through enough generations that there is a distinct and significant difference between the farmed cross types and the wild cross type with respect to their immune responses.

While 13 of the 17 GOIs tested were not influenced by cross type, at the expression level, four important immune relevant genes (i.e. *ifna*, *isg15a*, *rsad2a*, and *rsad2b*) were. In three of the four genes (except *rsad2b*), at least one farmed cross type showed significantly higher expression than the Wild cross type. This leads to the hypothesis that the farmed salmon have a stronger innate antiviral immune response than Wild salmon. This should be tested with live viruses as well as investigating the immune responses to bacterial infections. Another interesting result was that the expression levels of *isg15a*, *rsad2a*, and *rsad2b*, in Hyb.EO $_{W♀}$ were significantly higher than Wild, suggesting that Farm.EO plays a more important role than Wild in the direction of the cross producing the hybrid. This should be further investigated, again using live viruses, and again looking into the bacterial infections as well. Overall, the low number of cross type differences suggest that hybridization will not have a large effect on the innate antiviral-like immune response, but the fact that the four genes that do show a cross type difference are important to the immune response and suggest that there is some effect due to hybridization.

4.7 Conclusion

Overall, the results of this study demonstrate that there are differences among cross types in some immune relevant genes in response to IP injection with the viral-like PAMP pIC. With the exception of *stat1c*, *stat1a*, *mxb*, *pgds*, and *5loxb*, all other target genes were upregulated at 24 hpi, with a decline in induction at 72 hpi. Most cross type differences were found between pure cross types and unrelated hybrid cross types, supporting the idea that domestication genetically distinguishes wild and farmed populations as does their distinct origins (i.e. North American versus European). Also, the genetic implications suggest that the Wild cross type is the

least equipped to handle viral infections, while the Farm cross types and Hyb.NA $_{F♀}$ are the most equipped to handle viral infections. This suggest that hybridization may not negatively influence the immune response of wild populations. While this is a good thing, there are other concerns of hybridization that could be negatively affected such as aggression, feeding, risk aversion, stress related traits, to name a few. Such concerns can lead to the low survivorship of hybrids in the wild that has been frequently assessed (McGinnity et al., 2003; Skaala et al 2019; Sylvester et al., 2019).

The results of this study are based on a synthetic dsRNA analogue that elicits an antiviral-like response; it would be useful to do a similar study with live pathogens. There is also a need for future research on the implication of hybridization for general immunity (both innate and adaptive; as well as to both bacterial and viral pathogens). Moreover, the implications of such differences for performance/survival in the wild (i.e. going beyond a laboratory study) should be assessed.

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